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CONTENTS

Page

Further Developments in the Photometric Determination of Wheat Protein. *Lawrence Zeleny, M. H. Neustadt and H. B. Dixon* 1

The Influence of Temperature on the Development of Amylase in Germinating Wheat. *Eric Kneen, Byron S. Miller, and R. M. Sandstedt* 11

Amylase and Protease Systems of Malted Wheat Flour. *F. C. Hildebrand and G. M. Burkert*... 27

Nutritive Value of the Protein and Mineral Constituents of Rice Varieties. *A. Sreenivasan* 36

Nutritive Value of the Protein and Mineral Constituents of Dry and Wet Cultivated Rices. *A. Sreenivasan and V. Sadasivan* 47

The Riboflavin Content of Cereal Grains and Bread and Its Distribution in Products of Wheat Milling. *John S. Andrews, Harold M. Boyd, and David E. Terry* 55

On the Multiple Amylose Concept of Starch. II. Amylopectin and Amylose. *Ralph W. Kerr, O. R. Trubell, and G. M. Severson* 64

Quantitative Control Test for Iron Added to Flour. *J. T. Flohil and R. O. Brown* 81

The Relation of Fats to Texture, Crumb, and Volume of Bread. *J. C. Baker and M. D. Mize.* 84

Chemical Factors Affecting the Baking Quality of Dry Milk Solids. I. Correlation of pH and Baking Score. *U. S. Ashworth, N. S. Golding, G. H. Farrah, and D. D. Miller* 94

Factors Which Influence the Physical Properties of Dough. IV. The Effects of Surface Active Agents on the Characteristics of the Curves Made by the Recording Dough Mixer. *C. O. Swanson and A. C. Andrews* 102

Thiamin Retention in Self-Rising Flour Biscuits. *R. A. Barackman* 121

A Study of the Net Weight Changes and Moisture Content of Wheat Flour at Various Relative Humidities. *C. A. Anker and W. F. Geddes, with C. H. Bailey* 128

The Evaluation of Fluorophotometers to be Used in the Thiochrome Assay for Vitamin B_1. *John R. Loofbourow and Robert S. Harris* 151

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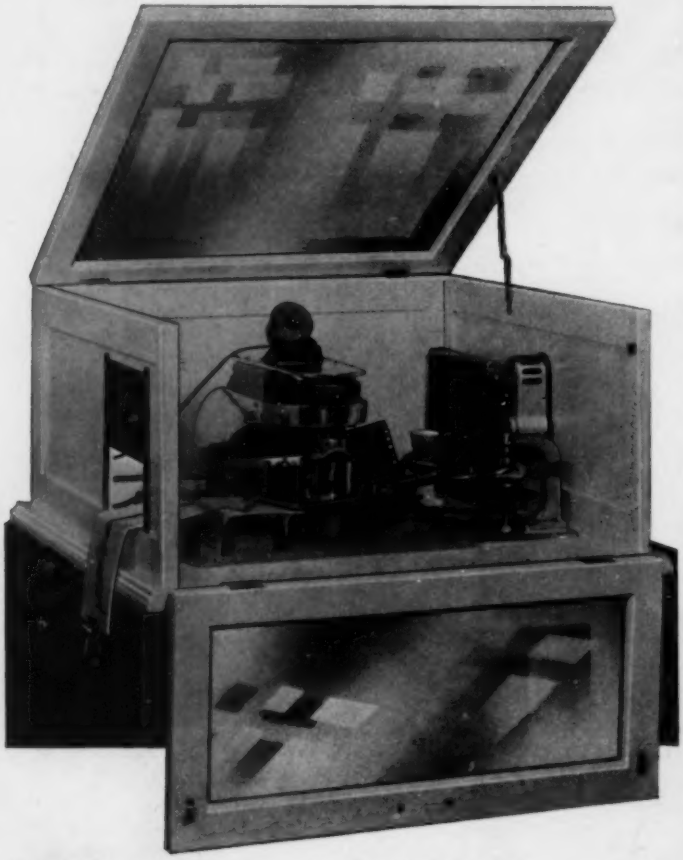

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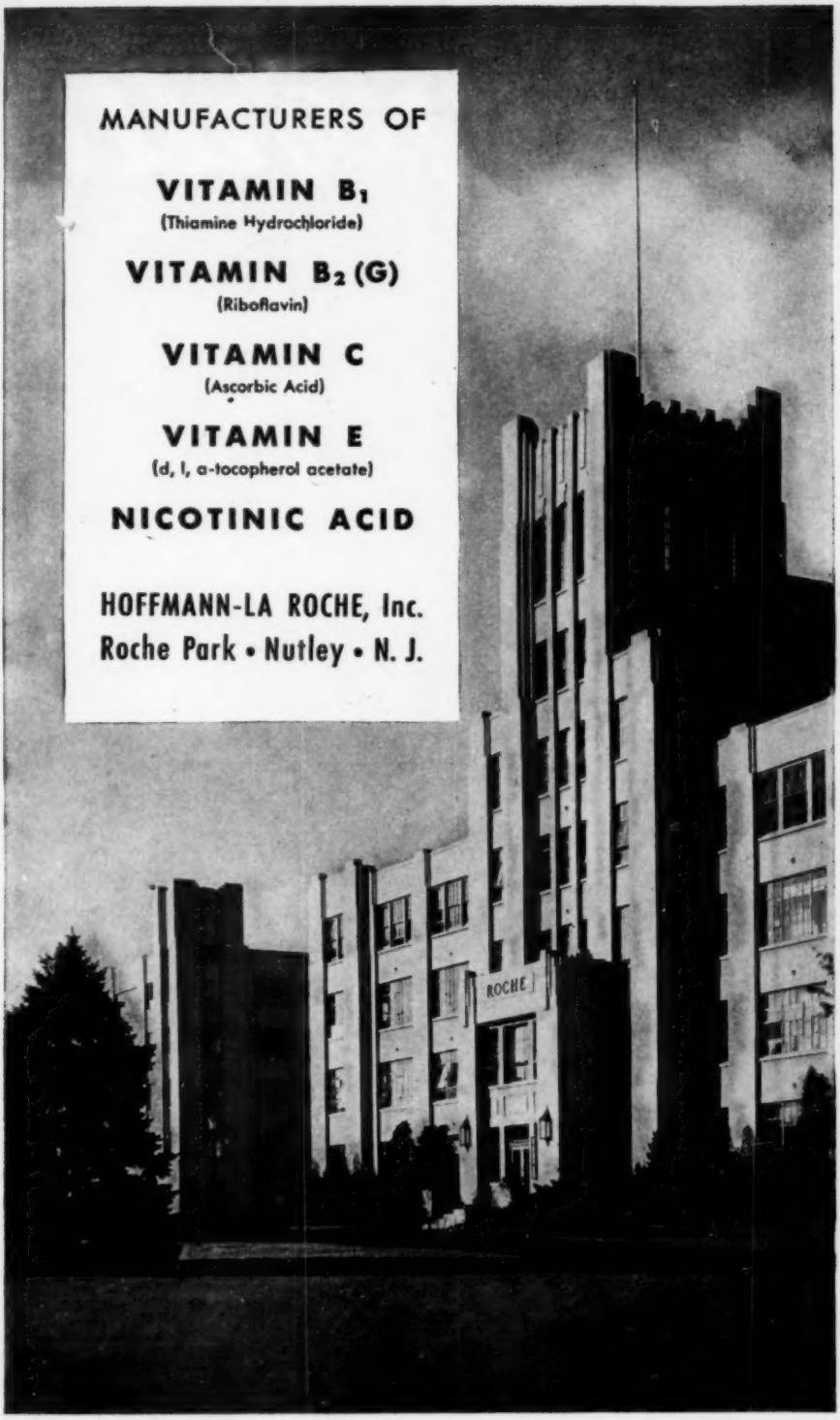

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CEREAL CHEMISTRY

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FURTHER DEVELOPMENTS IN THE PHOTOMETRIC DETERMINATION OF WHEAT PROTEIN

LAWRENCE ZELENY [1] M. H. NEUSTADT,[2] and H. B. DIXON [3]

Agricultural Marketing Service, U. S. Department of Agriculture, Washington, D. C.

(Read at the Annual Meeting, May 1941)

The photometric method for determining the protein content of wheat flour reported recently by one of us [4] has been subjected to further study by the Agricultural Marketing Service in an effort to develop a practical method, simpler and more convenient than the established Kjeldahl procedure, for determining protein in wheat. If the method could be developed in such a way that it would differentiate between the gluten proteins of the endosperm and the nongluten proteins of the nonendosperm constituents of the wheat kernel, its value would be greatly enhanced.

The method is based on the peptization of the wheat proteins by dilute alkali, the preparation of a stable colloidal suspension of the gluten proteins by accurately controlled partial neutralization of the alkaline extract, and the measurement of the light transmission through this suspension by means of the photoelectric photometer.

The proteins of ground wheat are readily peptized by dilute alkali. Neutralization of a portion of the alkaline extract to a definite pH by means of a suitable standard buffer solution causes a marked aggregation of the protein micelles of the prolamines and glutelins into particles sufficiently large to produce a marked turbidity but not large enough to cause precipitation within a reasonable length of time. Properly prepared suspensions of this type are remarkably stable and show very little change in optical density over a period of 24 hours. Globulins, albumins, proteoses, peptides, and amino acids remain completely dispersed under this treatment and, hence, do not contribute appreciably to the turbidity of the suspension.

Although wheat germ contains about 40% of protein, the protein consists chiefly of a globulin and an albumin, neither of which is deter-

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[4] L. Zeleny, A simple photometric method for determining the protein content of wheat flour, Cereal Chem. 18: 86–92 (1941).

mined by the photometric method. Bran contains about 17.5% protein, consisting of globulin and albumin in addition to considerable quantities of a prolamine and probably a glutelin. It might be expected that the latter two proteins could not be differentiated photometrically from the gluten proteins, but tests on relatively pure bran indicate that these proteins produce considerably less turbidity under the conditions of the test than do the corresponding proteins (the gluten proteins) of the endosperm. It may be stated, then, that the photometric method does differentiate in a large measure between the gluten and the nongluten proteins of the wheat and that the test is nearly, although not entirely, specific for the gluten proteins.

The Method

The photometric method used in this study was found to be applicable to both wheat and wheat flour. The details of the procedure are given below. This method differs in certain minor respects from the one previously reported for wheat flour (see footnote 4).

1. To exactly 0.5 g of the freshly and finely ground, well-mixed sample in a 130-ml centrifuge tube that can be stoppered, add 100 ml of 0.05*N* KOH solution.

2. Shake the stoppered tube intermittently for about 3 minutes, remove the stopper, and centrifuge for 10 minutes at approximately 1800 rpm. (In the case of flour lumps may form which must be completely broken up during the shaking process before centrifuging.)

3. To exactly 5 ml of the centrifugate in a photometer test tube (one of the selected tubes for use in lieu of an absorption cell), add exactly 25 ml of a buffer solution made by mixing 6 parts by volume of 0.2*M* KH_2PO_4 with 94 parts by volume of 0.2*M* Na_2HPO_4. This buffer solution should have a pH of 7.8 and should be preserved by the addition of 1 ml of toluene per liter of solution. Mix the contents of the test tube by inversion and allow to stand for 1 hour.

4. Determine the transmission of light through the solution in the test tube with a photoelectric photometer, using a light filter having a maximum transmission at a wave length of 530 millimicrons. (Other wave lengths will give different but equally satisfactory results.)

Determination of Endosperm Protein

Since the readings obtained by the photometer presumably are dependent upon the gluten protein rather than upon the total protein content of the sample and since the ratio of gluten protein to total protein varies widely among different samples of wheat, an accurate evaluation of the reliability of the photometric results as a measure of gluten protein can be made only by a direct comparison of photometric readings with gluten content values.

Unfortunately, however, there appears to be no direct and accurate method for determining the gluten protein content of wheat other than the extremely tedious and impractical procedure of dissecting the pure endosperm from a large number of kernels and carrying out peptization studies on the dissected material. In the absence of a practical direct method we have endeavored to calculate the endosperm protein content of wheat indirectly from the total protein and ash content of the sample. Although pure endosperm probably contains proteins other than the gluten proteins it is assumed that the content of such non-gluten protein in the endosperm is very small.

If the ash content of endosperm and the nonendosperm portion of a sample of wheat were known, the percentages of endosperm and non-endosperm constituents could be calculated by simple proportion using the equations:

$$N = \frac{100(A_W - A_E)}{A_N - A_E} \tag{1}$$

$$E = 100 - N \tag{2}$$

where $N = \%$ nonendosperm, $E = \%$ endosperm, $A_W = \%$ ash in wheat, $A_E = \%$ ash in endosperm, and $A_N = \%$ ash in nonendosperm.

Since pure bran and pure germ contain close to 9% and 5% of ash respectively and since the ratio of bran to germ in the wheat kernel is ordinarily about 13 : 2.5, the ash content of the nonendosperm portion of the wheat kernel is calculated to be approximately 8.4%. If it is assumed that the ash content of pure wheat endosperm is 0.35%, equation No. 1 may be simplified to:

$$N = \frac{100(A_W - 0.35)}{8.05} \tag{3}$$

Although the values for ash content of the endosperm and nonendosperm may vary from those used in equation No. 3, the difference between the two values is so great that the normal variability of either of them will have but little effect on the calculated value of N.

In the general run of hard red winter wheat the ratio of bran weight to germ weight may be considered to be reasonably constant, the weight of endosperm being the most variable factor. The range in wheat ash ordinarily encountered corresponds to a range in the non-endosperm constituents of from 10% to 30% as calculated from equation No. 3, and the range in germ content is generally considered to be from 2% to 3% of the wheat. Using these values, the germ content and the bran content of any sample may be calculated from the non-endosperm content by means of the following equations:

$$G = 0.05N + 1.5 \tag{4}$$

and

$$B = N - G \tag{5}$$

where G = % germ, B = % bran, and N = % nonendosperm (from equation No. 3).

Since pure bran is said to contain approximately 17.5% protein and pure germ 40% protein, the bran and germ protein contents of the original wheat may be determined by using the values obtained in equations No. 4 and No. 5. The endosperm protein is then obtained by subtracting the sum of the bran and germ protein from the total protein value.

The above method for determining the endosperm protein content of wheat is based on the assumption that the variation in ash content among different samples of wheat is due to the variation in the proportions of the three major structures of the wheat kernel, endosperm, germ, and bran, and not to a variation in the ash content of the individual structures themselves. It is also based on the assumption that variation in wheat protein is due primarily to variation in relative size and protein content of the endosperm, and that the protein content of pure bran and of pure germ is relatively constant. Obviously these assumptions are not strictly true and the calculated values for endosperm protein cannot, therefore, be considered highly accurate. Except in unusual instances, however, it is not likely that variabilities in the values assumed to be constant are sufficiently great to cause serious errors in this method of calculation.

Experimental Work

One hundred and ninety-five samples of commercial hard red winter wheat of the 1940 crop from 15 different states were analyzed by the photometric method and the light transmission values compared with the protein content values determined by the Kjeldahl method. The results are listed in Table I and are shown graphically in Figure I.

From a study of the graphical presentation of these data (Fig. 1) it became evident that in the case of wheats that were characterized by low test weight, high ash content, and a shriveled appearance, light transmission values usually indicated lower protein contents than those shown by Kjeldahl analysis. Such wheats contain more bran and less endosperm than normal wheats and consequently show a lower than average ratio of gluten protein to total protein. Conversely in the case of wheats that were characterized by high test weight, low ash content, and a plump appearance, light transmission values usually indicated higher protein contents than those shown by Kjeldahl analysis. Wheats of this type contain less bran and more endosperm than average wheats and hence show a higher than average ratio of gluten protein to total protein. These observations support the theory

TABLE I

TOTAL PROTEIN, ASH, ENDOSPERM PROTEIN (CALCULATED), AND LIGHT TRANSMISSION VALUES ON 195 SAMPLES OF HARD RED WINTER WHEAT FROM THE 1940 CROP

(Samples are listed in order of increasing total protein content. All data are on an "as is" moisture basis.)

Sample No.	State	Total protein (Kjeldahl)	Ash	Endosperm protein	Light transmission
		%	%	%	%
137	Idaho	8.23	1.47	5.30	76.5
455	Washington	8.28	1.62	5.00	75.2
439	Washington	8.38	1.35	5.72	72.2
621	Washington	8.53	1.62	5.25	73.4
540	Montana	8.62	1.76	5.00	76.3
1000	Indiana	8.66	1.82	4.92	73.0
614	Washington	8.73	1.48	5.77	70.3
139	Idaho	8.80	1.72	5.27	72.9
145	Washington	8.96	1.68	5.54	73.2
147	Idaho	9.04	1.54	5.95	73.0
148	Washington	9.05	1.62	5.77	72.6
858	Montana	9.06	1.77	5.42	73.5
479	Washington	9.10	1.68	5.86	71.8
433	Washington	9.33	1.46	6.41	69.9
480	Idaho	9.38	1.78	5.72	69.5
489	Idaho	9.38	1.76	5.76	69.5
163	Washington	9.43	1.58	6.22	69.3
451	Montana	9.48	1.68	6.06	70.3
486	Washington	9.50	1.73	5.95	69.6
475	Washington	9.52	1.62	6.24	68.7
985	Indiana	9.53	1.75	5.94	69.8
806	Kansas	9.53	1.74	5.96	71.5
143	Washington	9.53	1.61	6.28	69.9
443	Washington	9.55	1.70	6.09	69.3
173	Washington	9.64	1.60	6.39	68.2
155	Washington	9.64	1.39	6.91	67.9
628	Washington	9.68	1.65	6.33	71.3
456	Washington	9.68	1.58	6.47	71.1
135	Washington	9.70	1.61	6.45	70.5
171	Washington	9.71	1.62	6.43	69.8
437	Washington	9.75	1.47	6.82	70.5
129	Washington	9.76	1.79	6.09	72.6
141	Washington	9.77	1.64	6.45	69.5
612	Washington	9.78	1.49	6.80	68.2
854	Montana	9.79	1.70	6.33	69.8
374	Illinois	9.79	1.68	6.37	70.1
631	Washington	9.81	1.48	6.85	68.6
467	Washington	9.86	1.58	6.65	70.2
385	Illinois	9.87	1.77	6.23	69.4
453	Washington	9.92	1.42	7.10	68.1
436	Washington	9.93	1.59	6.72	66.7
617	Washington	9.93	1.46	7.01	67.1
472	Washington	9.96	1.45	7.07	69.3
174	Idaho	9.97	1.79	6.30	70.0
161	Washington	10.04	1.35	7.38	67.2
447	Oregon	10.05	1.95	6.00	70.0
444	Washington	10.07	1.59	6.86	70.6
756	Kansas	10.11	1.56	6.99	68.5
694	Illinois	10.25	1.89	6.33	71.3
718	Illinois	10.35	1.93	6.34	69.9
478	Washington	10.42	1.50	7.42	65.9
672	Illinois	10.46	1.71	6.98	69.4

TABLE I—*Continued*

Sample No.	State	Total protein (Kjeldahl)	Ash	Endosperm protein	Light transmission
		%	%	%	%
857	Minnesota	10.57	2.08	6.20	66.5
998	Indiana	10.62	1.87	6.77	67.9
562	Kansas	10.62	1.77	6.98	68.3
997	Indiana	10.67	1.84	6.87	68.9
673	Illinois	10.70	1.81	6.97	68.5
737	Kansas	10.89	1.49	7.91	64.5
675	Illinois	11.10	1.84	7.30	66.8
715	Illinois	11.22	1.87	7.37	68.2
671	Illinois	11.23	1.74	7.66	65.5
595	Kansas	11.27	1.78	7.61	64.8
609	Kansas	11.32	1.76	7.70	63.2
681	Illinois	11.32	1.71	7.84	68.4
683	Missouri	11.38	1.87	7.53	66.7
674	Illinois	11.41	1.81	7.68	66.9
682	Illinois	11.46	1.83	7.70	65.6
984	Illinois	11.47	1.81	7.74	64.2
600	Kansas	11.52	2.02	7.32	64.5
865	Iowa	11.52	1.86	7.69	68.7
844	Montana	11.56	1.67	8.15	62.9
680	Illinois	11.58	1.77	7.94	65.0
869	Iowa	11.59	1.88	7.72	64.4
702	Kansas	11.60	1.66	8.23	62.3
729	Kansas	11.61	1.59	8.40	63.1
676	Oklahoma	11.72	1.78	8.06	63.8
748	Oklahoma	11.77	1.85	7.96	65.2
866	Iowa	11.80	2.05	7.54	64.4
592	Kansas	11.80	1.65	8.45	61.5
678	Illinois	11.83	1.78	8.17	63.9
1001	Iowa	11.91	1.62	8.63	62.2
571	Nebraska	11.92	1.74	8.35	63.8
632	Oregon	12.03	1.77	8.39	63.8
584	Kansas	12.04	1.66	8.67	60.8
604	Kansas	12.04	1.57	8.86	61.4
665	Oregon	12.07	1.76	8.45	62.9
579	Kansas	12.12	1.77	8.48	63.6
961	Oklahoma	12.12	1.78	8.46	61.8
846	Montana	12.21	1.57	9.03	62.3
606	Kansas	12.28	1.88	8.41	60.0
599	Kansas	12.30	1.63	9.00	59.9
589	Nebraska	12.40	1.81	8.67	61.2
711	Kansas	12.42	1.84	8.62	60.8
835	Iowa	12.47	1.76	8.85	61.4
567	Kansas	12.48	1.70	9.02	61.0
1086	Texas	12.54	1.73	8.99	62.5
789	Iowa	12.71	1.89	8.79	62.2
561	Kansas	12.75	1.78	9.09	59.9
629	Montana	12.76	1.58	9.55	61.0
825	Iowa	12.81	1.92	8.82	61.6
713	Oklahoma	12.86	1.76	9.24	59.2
588	Oklahoma	12.87	1.80	9.18	59.0
620	Montana	12.91	1.62	9.63	59.2
828	Iowa	12.92	1.96	8.86	61.7
568	Kansas	13.26	1.63	9.96	57.4
586	Kansas	13.37	1.78	9.71	57.9
861	Iowa	13.49	1.77	9.85	58.3
952	Oklahoma	13.62	1.69	10.18	55.1

TABLE I—*Continued*

Sample No.	State	Total protein (Kjeldahl)	Ash	Endosperm protein	Light transmission
		%	%	%	%
1031	Iowa	13.62	1.71	10.14	55.9
686	Oklahoma	13.68	1.61	10.43	56.0
692	Oklahoma	13.73	1.88	9.86	56.8
630	Montana	13.76	1.49	10.78	56.6
940	Olkahoma	13.87	1.74	10.30	57.8
573	Kansas	13.89	1.81	10.16	58.4
685	Oklahoma	13.97	1.82	10.23	54.4
615	Montana	14.09	1.46	11.17	53.7
623	Montana	14.11	1.50	11.11	56.7
618	Montana	14.16	1.56	11.04	53.8
613	Montana	14.26	1.39	11.53	53.4
785	Nebraska	14.32	1.76	10.70	55.5
684	Oklahoma	14.37	1.92	10.38	56.9
873	Wyoming	14.37	1.70	10.91	56.7
622	Montana	14.47	1.67	11.06	53.9
791	Nebraska	14.51	1.84	10.71	56.7
605	Kansas	14.51	1.73	10.96	52.5
783	Nebraska	14.56	1.88	10.69	56.9
788	Nebraska	14.56	1.77	10.92	52.6
601	Kansas	14.56	1.65	11.21	55.7
624	Montana	14.69	1.58	11.48	52.2
627	Montana	14.71	1.59	11.50	57.2
790	Nebraska	14.78	1.75	11.19	55.3
559	Nebraska	14.80	1.78	11.14	53.9
578	Kansas	14.87	1.89	10.95	54.3
616	Montana	14.88	1.35	12.22	49.2
784	Nebraska	14.92	1.92	10.93	56.2
843	Montana	14.96	1.66	11.59	51.7
572	Kansas	15.06	1.76	11.44	56.4
574	Oklahoma	15.12	1.76	11.55	51.3
677	Oklahoma	15.13	1.89	11.21	52.2
564	Nebraska	15.20	1.77	11.56	53.3
747	Texas	15.21	1.86	11.38	52.1
786	Nebraska	15.34	1.79	11.67	52.2
745	Texas	15.35	2.02	11.15	52.6
679	Texas	15.36	1.99	11.23	50.2
587	Kansas	15.56	2.06	11.25	54.6
602	Nebraska	15.56	1.98	11.44	53.3
590	Kansas	15.58	1.83	11.82	50.4
576	Kansas	15.66	1.65	12.31	50.8
619	Montana	15.66	1.47	12.73	50.7
734	Oklahoma	15.69	1.73	12.14	52.3
591	Nebraska	15.71	1.70	12.25	49.0
625	Montana	15.74	1.59	12.53	49.9
913	Wyoming	15.76	1.69	12.32	50.7
585	Kansas	16.09	1.86	12.26	50.6
607	Kansas	16.31	2.17	11.77	51.2
691	Oklahoma	16.38	1.71	12.90	48.6
603	Kansas	16.41	2.01	12.22	50.3
693	Oklahoma	16.51	1.86	12.68	49.1
626	Montana	16.51	1.61	13.26	51.5
582	Kansas	16.54	2.19	11.95	52.6
593	Nebraska	16.63	2.04	12.39	49.4
575	Kansas	16.76	2.25	12.02	49.9
580	Nebraska	16.81	2.19	12.22	51.0

TABLE I—*Continued*

Sample No.	State	Total protein (Kjeldahl)	Ash	Endosperm protein	Light transmission
		%	%	%	%
849	Colorado	16.83	1.94	12.80	48.7
594	Kansas	16.84	1.83	13.08	48.9
610	Montana	16.91	1.61	13.66	48.7
577	Kansas	16.94	2.10	12.54	48.3
787	Nebraska	16.99	1.94	12.96	48.8
1085	Texas	17.00	1.88	13.13	46.9
896	Wyoming	17.16	1.89	13.24	50.3
1078	Texas	17.36	2.03	13.14	45.5
797	Kansas	17.39	2.22	12.70	47.7
583	Kansas	17.40	2.14	12.91	50.1
581	Nebraska	17.41	2.06	13.10	48.5
565	Kansas	17.47	2.03	13.25	48.0
569	Kansas	17.68	2.21	13.03	46.1
560	Nebraska	17.74	2.18	13.18	49.5
608	Nebraska	17.91	2.24	13.19	45.7
598	Kansas	17.98	2.25	13.24	48.1
570	Kansas	17.99	2.18	13.43	48.5
527	Colorado	18.03	2.17	13.49	45.8
566	Nebraska	18.04	2.03	13.82	45.9
543	Kansas	18.15	2.33	13.25	47.6
596	Kansas	18.19	2.08	13.82	45.2
518	Kansas	18.22	2.29	13.39	46.5
563	Kansas	18.32	2.49	13.03	47.8
597	Kansas	18.32	2.25	13.58	47.4
58	Kansas	18.36	2.34	13.45	46.1
28	Kansas	18.45	2.32	13.57	43.3
23	Kansas	18.48	2.15	13.97	44.2
72	Kansas	18.65	2.33	13.75	43.6
11	Kansas	18.65	2.32	13.77	43.3
84	Kansas	18.71	2.26	13.94	45.2
40	Kansas	18.76	2.54	13.39	47.1
59	Kansas	18.82	2.13	14.35	44.5

that light transmission values are a better index of gluten protein than of total protein content.

Approximate values of endosperm protein content for the series of samples under investigation were determined by the method herein described. These values also are listed in Table I and are shown graphically in Figure 2. A comparison of Figures 1 and 2 indicates that the endosperm protein appears to bear a linear relationship to the light transmission but that the relationship between total protein and light transmission is curvilinear. For the purposes of statistical analysis it was found that this latter relationship could be made linear by using the arcsine of the total protein content rather than the total protein content itself.[5]

The following statistical values for correlation coefficient and standard errors of estimate were obtained:

[5] For a discussion of this technique see G. W. Snedecor, Statistical methods (3rd ed.), p. 382 (1940), the Iowa State College Press.

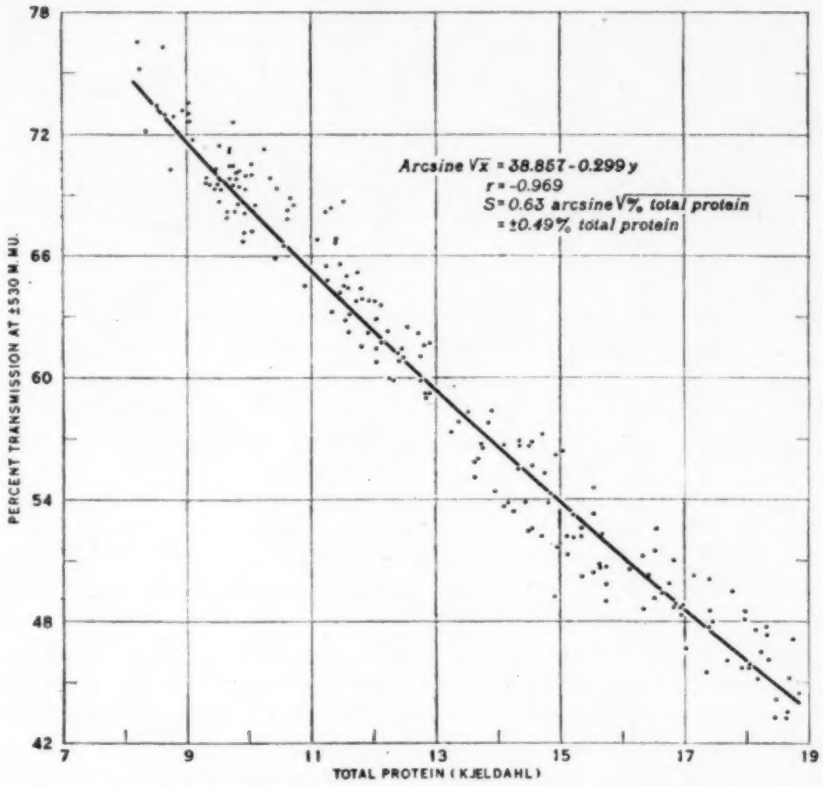

Fig. 1. Total protein and light transmission values on 195 samples of hard red winter wheat.

For total protein and light transmission

$r = -0.969$

$S = 0.634$ arcsine of the total protein percentage or approximately 0.49% total protein.

For endosperm protein and light transmission

$r = -0.987$

$S = 0.44\%$ endosperm protein

The further observation was made that in the case of the endosperm protein data (Fig. 2) 76% of the values fell within 0.5% (in terms of endosperm protein) of the regression line, while in the case of the total protein data (Fig. 1), only 62% of the values fell within 0.5% of the theoretical curve representing the relationship between total light transmission and total protein. Thus the light transmission values are shown to be a somewhat better measure of endosperm protein (and therefore presumably of gluten protein) than of total protein content. This observation lends further support to our theoretical contention.

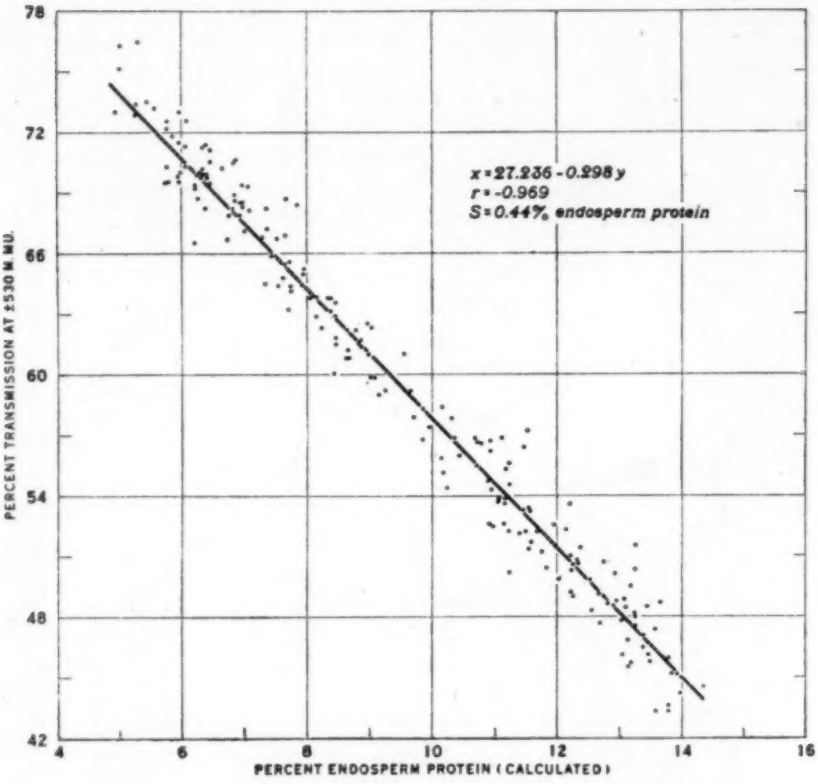

Fig. 2. Endosperm protein (calculated) and light transmission values on 195 samples of hard red winter wheat.

Summary

The photometric method previously reported for the determination of protein in wheat flour has been applied, with minor modifications, to the analysis of wheat. Theoretically the method differentiates to a considerable degree between gluten and nongluten protein and thus comes closer to being a measure of gluten protein than of total protein. Experimental evidence substantiating this theory is presented.

The principal advantages of the photometric method for routine protein analysis should be the ease and rapidity with which a large volume of work can be handled with relatively simple equipment and without the unpleasant features usually associated with a protein laboratory. An additional advantage is that the results obtained are probably a somewhat better index of ultimate baking quality than are the values obtained by the conventional Kjeldahl procedure.

Further work will be directed toward the adequate standardization of the method and of the photometers used in order that concordant results may be obtained among different laboratories.

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THE INFLUENCE OF TEMPERATURE ON THE DEVELOPMENT OF AMYLASE IN GERMINATING WHEAT [1]

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The importance of amylolytic action in the brewing, distilling, and other industries and in the supplementation of bread flours has stimulated much investigation of the development of amylase during the germination of cereal grains. Early studies were mainly evaluations of the increase in saccharogenic power during the production of malt. The work of Lintner (1887) and Brown and Morris (1890) did much to clarify the conception of amylase development during malting. These authors found that malt diastatic power was the result of the action of two components: a sugar-producing enzyme similar to that in the original ungerminated grain and a "liquefying" enzyme appearing during germination. Through the following years the "two enzyme" theory developed but it was not until the work of Ohlsson (1926, 1930) that a means was provided for following the development of the individual amylase components, alpha- (dextrinogenic) and beta- (saccharogenic) amylase, during germination of grain. Ohlsson and coworkers (Nordh and Ohlsson, 1932; Ohlsson and Uddenberg, 1933; Ohlsson and Edfeldt, 1933; Stenstam, Björling, and Ohlsson, 1934; Ohlsson and Thörn, 1938), Lüers and Rümmler (1935), and Ugrümow (1935) used this differential inactivation technique (70°C to inactivate beta-amylase, pH 3.3 to inactivate alpha-amylase) to study the elaboration of these enzymes during germination of barley, wheat, rye, and oats. Their researches indicate that beta-amylase activity increases in the early stages of seedling development and attains a maximum after a few days, and that alpha-amylase activity appears

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after a day or so of germination and increases throughout the germination period.

Following a somewhat different approach, many investigations have been made on the nature of the increase in saccharogenic power during germination. The work of Ford and Guthrie (1908a, b) demonstrated that the action of proteolytic enzymes liberated additional saccharogenic amylase from ungerminated barley and wheat. The researches of several authors, notably Weichherz and Asmus (1931), Waldschmidt-Leitz and Purr (1931), Myrbäck and Myrbäck (1933), Lüers and Lechner (1933), Chrzaszcz and Janicki (1933) and Lüers and Rümmler (1933), resulted in the conclusion that the increase in saccharifying activity coincident with germination is largely the result of the liberation of the "bound" amylase of the ungerminated cereal by the action of the proteolytic enzymes simultaneously developing during germination. More recent work of Hills and Bailey (1938a, b) and of Snider (1941) indicates that malt saccharogenic activity is dependent upon the combined action of the beta-amylase, that originally present in the "free" state in grain plus that liberated by proteolytic action during malting, and the alpha-amylase formed or activated in the germination process.

The literature [2] provides no data relating exclusively to the development of the *individual* amylase components during germination. The importance of considering the activities of these individual components in malt studies has recently been emphasized by Kneen, Beckord, and Sandstedt (1941). Further, Kneen and Sandstedt (1941b) clearly demonstrate that the value of malt as a flour supplement is dependent upon the content of only one component, alpha-amylase. It is important then to re-examine the nature of the development of the amylases during the germination of cereal grains.

The Ohlsson (1930) technique of differential inactivation of the amylases is at best only qualitative. The heat treatment results in the loss of a portion of the alpha component, while the acid treatment not only inactivates a part of the beta component but rarely effects complete inactivation of alpha-amylase. Recent methods of Sandstedt, Kneen, and Blish (1939) and Kneen and Sandstedt (1941a) have made practical the determination of these individual components without such differential inactivation. The purpose of the present study was to use these methods to measure accurately the development of the amylase components in germinating wheat as influenced by germination temperature.

[2] Additional literature review may be found in the articles by Lüers (1936) and Weichherz and Asmus (1931).

Methods

Preparation of wheat: A sample of hard red winter wheat was carefully sorted and all broken or badly shriveled kernels removed. Twelve samples having 500 seeds each were counted out and weighed. The average weight per 1,000 kernels was found to be 20.06 g. Samples used for germination were 30 g each or approximately 1,500 seeds. No seed treatment other than the 12-hour pregermination soak was used.

Germination: The 30-g samples of wheat were soaked in distilled water for 12 hours at room temperature and then transferred to "rag dolls" [3] for germination. The dolls were immediately placed in germination cabinets at the desired temperature. Each doll contained a 30-g sample of wheat and a sufficient number were maintained at each temperature so that the contents of a whole doll were available for each analysis. Four cabinets were used, each being held at constant temperature throughout the experiment. The germination temperatures used were 20°, 15°, 10°, and 5°C. Humidity was kept at a high level in all cabinets. This method of germination proved particularly desirable for enzyme studies during germination of seeds not previously given a fungicidal treatment. Some mold growth did appear towards the last of the germination period in the dolls held at 20°C and at 15°C, but none was apparent at any time at 10° or 5°C. The few moldy seedlings occurring were removed after the samples were weighed and dried.

Sampling: At the end of the desired period of germination (measured from the time the dolls were placed in the germinator) the contents of a rag doll were removed quantitatively to a moisture can and total green weight determined. Immediately following the weighing, a sample was taken for the determination of dry-matter content. The remainder was spread out thinly under high-speed fans and dried at room temperature. An estimate of sprout length was made at this time. When the sample was dry it was ground finely in a burr mill and samples taken for dry-matter determination and for extraction.

Extraction: Two types of extracts were prepared, (1) a "free" extract resulting from a 3-hour extraction at 30°C of 5 g of the meal with 50 ml of dilute calcium acetate solution (1 mg calcium acetate per 1 ml) and (2) a total extract resulting from an 18-hour extraction at 30°C of 5 g of meal plus 0.2 g of Merck's papain with 50 ml of the dilute calcium acetate solution. These extracts were diluted to concentrations adapted to the determinations of saccharifying and alpha-dextrinizing activities. It was realized that the choice of a 3-hour

[3] The modified rag doll commonly used in agronomic practice for germinating corn is described by Duddleston (1920).

period for the "free" and an 18-hour period for the papain extraction was arbitrary. These times roughly correspond to those used by other workers. It is doubtful if *any* water extract of malt can properly be termed a free extract, since liberation of the "bound" amylase by the proteolytic enzymes present in the germinated grain itself is initiated as soon as extraction commences. However, these techniques do give some idea as to the relative availability of the amylases in different samples. The papain used was of adequate activity; greater quantities or activation treatments resulted in no greater extraction of the amylases.

Determination of amylase activities: Saccharogenic activity and beta-saccharogenic activity were determined according to the technique of Kneen and Sandstedt (1941a) and are reported as saccharogenic and beta-amylase units respectively. Alpha-dextrinogenic activity on those samples having more than two units of alpha-amylase activity was determined by the method of Sandstedt, Kneen, and Blish (1939). With those samples having less than two units of activity the method of Sandstedt and Kneen (1941) for the determination of small quantities of alpha-amylase was used. This "micro method" for alpha-amylase consists essentially of allowing the extract to act on the buffered alpha-amylodextrin solution at 30°C for some 18 hours. At the end of this period a known amount of alpha-amylase is added and the time taken to reach the standard end point with iodine is determined. The difference between this dextrinization time and that for the known amount of alpha-amylase is a measure of the degree of dextrinization attributable to the small quantity of alpha-amylase present in the unknown extract. As in the "macro method" alpha-amylase activity is expressed as "the number of grams of soluble starch which, under the influence of an excess of beta-amylase, are dextrinized by one gram of malt in one hour at 30°C."

Extracts equivalent to 0.01 g of malt were found satisfactory for all the determinations of saccharogenic activity. As seedling growth progressed it was found advisable to vary the weights used for the determination of alpha-amylase activity. In the "micro method" these weight equivalents varied from 0.2 g of the ungerminated grain to 0.01 g of a malt having approximately two units of alpha-amylase activity. In the "macro method" weight equivalents ranged from 0.2 to 0.01 g of malt.

Results

All the data pertinent to the germination study are given in Table I. This table is divided into four sections to cover the results of germination at the four temperatures, 20°, 15°, 10°, and 5°C. For each germination period data are presented for total green and total dry weight

TABLE I

WEIGHT CHANGES, SPROUT ELONGATION, AND AMYLASE DEVELOPMENT OF WHEAT GERMINATING AT THE FOUR TEMPERATURES OF 20°, 15°, 10°, AND 5°C

Days germinated	Total green weight	Total dry weight	Sprout length	Saccharogenic activity		Beta-amylase activity		Alpha-amylase activity	
				"Free"	Total	"Free"	Total	"Free"	Total
	g	*g*	*mm*	*units*	*units*	*units*	*units*	*units*	*units*
GERMINATION TEMPERATURE: 20°C									
0	30.0	26.7	—	10.1	28.4	10.1	28.4	0.027	0.043
½	47.7	27.8	—	11.0	28.6	11.0	28.6	0.351	0.359
1	49.1	27.2	1–2	12.8	29.3	12.7	29.2	1.83	1.95
1½	50.4	27.0	2–3	14.2	28.6	13.8	28.1	8.50	9.40
2	53.5	27.1	3–4	17.3	28.5	16.1	27.2	24.0	23.8
2½	57.1	27.2	5–7	22.2	31.4	19.3	28.5	55.6	56.9
3	55.1	25.1	6–9	25.2	32.0	21.5	28.1	72.8	79.4
3½	62.4	21.8	10–20	30.1	35.3	23.3	27.6	138	153
4	64.7	19.0	20–30	37.7	39.1	26.0	27.2	238	242
GERMINATION TEMPERATURE: 15°C									
0	30.0	26.7	—	10.1	28.4	10.1	28.4	0.027	0.043
½	47.2	27.7	—	10.7	29.3	10.7	29.3	0.097	0.122
1	48.1	27.2	0–1	11.9	29.3	11.9	29.3	0.595	0.546
1½	49.2	27.3	½–2	11.8	28.2	11.7	28.1	1.87	2.34
2	50.7	26.9	1–2	13.3	28.3	13.0	28.0	6.00	6.37
2½	52.2	27.4	2–3	16.9	31.6	16.0	30.7	18.1	18.7
3	54.7	27.1	3–5	18.1	30.6	16.3	28.6	35.0	40.5
4	61.8	27.0	5–10	25.1	31.1	20.8	26.5	87.2	90.7
5	78.8	25.8	20–25	37.9	37.9	26.6	26.2	229	238
6	89.5	26.5	20–30	39.1	39.0	26.9	26.3	250	260
GERMINATION TEMPERATURE: 10°C									
0	30.0	26.7	—	10.1	28.4	10.1	28.4	0.027	0.043
1	47.6	27.3	—	11.6	29.8	11.6	29.8	0.170	0.120
2	49.3	27.3	—	12.1	30.9	12.1	30.9	0.918	1.02
3	51.6	27.0	1–2	13.0	30.7	12.7	30.4	5.22	5.68
4	53.1	26.8	2–4	17.3	30.4	16.0	28.8	26.6	31.2
5	55.8	26.9	3–5	20.1	31.9	17.7	29.3	47.6	51.3
6	60.0	27.2	5–6	24.4	34.4	20.3	30.2	80.6	83.2
7	65.7	26.1	7–10	28.0	36.4	22.3	29.5	114	134
8	73.9	26.1	10–14	30.9	37.3	23.4	28.8	148	167
9	88.1	25.3	15–25	35.0	38.0	24.9	27.3	205	217
10	99.2	25.7	25–35	41.1	40.7	28.2	27.2	265	281
GERMINATION TEMPERATURE: 5°C									
0	30.0	26.7	—	10.1	28.4	10.1	28.4	0.027	0.043
1	47.7	28.4	—	10.9	28.3	10.9	28.3	0.080	0.082
2	48.1	27.1	—	11.8	29.4	11.8	29.4	0.222	0.375
3	49.3	26.9	0–1	9.5	29.3	9.5	29.3	0.780	0.800
4	49.8	26.8	½–1	8.9	29.5	8.9	29.4	1.20	1.72
5	50.6	27.2	1–1½	9.3	30.4	9.2	30.2	2.34	4.07
6	50.7	27.0	1–2	12.9	30.1	12.6	29.8	6.11	6.24
8	52.5	26.7	1–3	13.2	32.3	12.5	31.5	14.1	15.8
10	53.5	26.8	1–3	14.0	31.1	12.9	29.7	22.2	26.8
12	55.4	26.4	2–3	17.9	31.1	16.0	28.7	37.9	48.5
16	60.9	26.1	4–7	23.0	34.4	18.5	29.6	92.2	94.5
20	74.4	25.9	12–17	34.0	37.9	25.2	28.8	177	182
24	95.5	22.7	25–35	40.3	40.8	27.9	27.9	258	265

of the sample, for approximate sprout length, and for the various amylolytic activities of both the "free" and the total (papain) extracts. Saccharogenic and beta-amylase units are those of Kneen and Sandstedt (1941a) and alpha-amylase units those of Sandstedt, Kneen, and Blish (1939). In all instances then the amylase activity is referred to as the starch hydrolysis caused by one gram of the dry meal.

Germination at each temperature was terminated when sprout length reached 30 to 35 mm. For adequate data it was necessary to sample every 12 hours at the 20° germination temperature and at least daily at 15° and 10°C. On the other hand germination progressed slowly at 5° and sampling was not done so frequently. It should be re-emphasized that a germination period of one day indicates one day in the rag doll *after* soaking and that the data of Table I refer to the whole seedling.

Seedling weight and sprout length: Gaumann (1932), in extensive research on the germination of wheat, found that velocity of growth was more rapid and loss of dry weight more pronounced at high temperatures than at low. The data of Table I support these findings. At 20°, 15° 10°, and 5°C, the times necessary to produce sprouts of from 30 to 35 mm length were respectively 4, 6, 10, and 24 days. At the end of the germination periods the green weights of the 15°, 10°, and 5° samples were triple that of the original grain. However, at 20° the original weight was only doubled. Very little loss of dry weight occurred at either 15° or 10°C. Some loss was apparent during the long period at 5°C and a very decided loss during the four days at 20°C. This rapid loss in dry weight at high temperatures of germination, combined with the tendency of the sample to dry out and to acquire mold growth, made germination at 20°C a rather unsatisfactory procedure.

Influence of pregermination treatment: Ohlsson and co-workers (1932-1938) included the period of soaking (as long as 3 days) in the germination period. Stenstam, Björling, and Ohlsson (1934) reported that, in germinating wheat, the extractable beta-amylase decreased during this soaking treatment. Their macro method for alpha-amylase indicated an absence of this component during the preliminary treatment. To investigate any possible changes taking place during the pregermination treatment, samples soaked for 12 and 24 hours were included in the analysis. Table II shows the results obtained. Data for the untreated wheat sample and for 12 hours of germination at 15°C are included for comparison. The data indicate that changes in extractable beta-amylase, either free or total, are insignificant during soaking. However, this treatment does seem to result in some increase in both free and total alpha-amylase, particularly during the first 12 hours of soaking.

Since the soak water was always drained off and discarded before the samples were transferred to rag dolls, amylase activity in this discarded portion was determined. The methods used indicated that beta-amylase activity was absent and that only a trace of alpha-amylase activity could be detected. The pregermination soak resulted then in an absorption of water, no significant change in beta-amylase, and some increase in alpha-amylase.

TABLE II

WEIGHT CHANGES AND AMYLASE DEVELOPMENT IN WHEAT DURING THE PREGERMINATION SOAK

Treatment	Total green weight	Total dry weight	Beta-amylase activity		Alpha-amylase activity	
			"Free"	Total	"Free"	Total
	g	*g*	*units*	*units*	*units*	*units*
Untreated	30.0	26.7	10.1	28.4	0.027	0.043
12-hour soak	39.4	27.4	9.7	28.9	0.035	0.070
24-hour soak	45.8	26.9	10.6	28.8	0.029	0.056
12-hour germination	47.2	27.7	10.7	29.3	0.097	0.122

Saccharogenic activity: Curves indicating the development of saccharogenic activity during germination at the four temperatures are shown in Figure 1. It may be noted from Table I that in the instances of germination at 20°, 15°, and 10°C the total saccharogenic activity showed an increase during the initial period, followed in turn by a slight decrease and then a steady increase. The significance of these minor variations is questionable and to prevent confusion in the graphing such initial deviations are not shown by the curves of Figure 1. Likewise the same behavior may be noted in connection with the free extracts of those samples germinated at 5°C. Here, too, the variation is of questionable significance and was ignored in drawing the curve.

The data, as recorded in Figure 1, clearly illustrate the manner in which saccharifying activity, both free and total, increases slowly during the initial stages and then at an accelerated rate as the seedling develops. This is true at all four germination temperatures. The amounts of free and total activity approach each other and are approximately equal at the level of about 40 units. This level was reached at about 4, 6, 10, and 24 days respectively for the four germination temperatures of 20°, 15°, 10°, and 5°C.

The data of Figure 1 are in agreement with the findings of Weichherz and Asmus (1931) and Myrbäck and Myrbäck (1933) that the free and total saccharogenic activities of germinating barley increased steadily and became approximately equal in the later stages of germination. They do not agree with the findings of Effront (1905), Wald-

schmidt-Leitz and Purr (1931), and Lüers and Rümmler (1933) for barley, and Naylor and Dawson (1936) for oats, that saccharifying power decreases in the later stages of seedling development. The enzymatic changes responsible for the development of saccharogenic activity are best illustrated by a consideration of the individual amylase components.

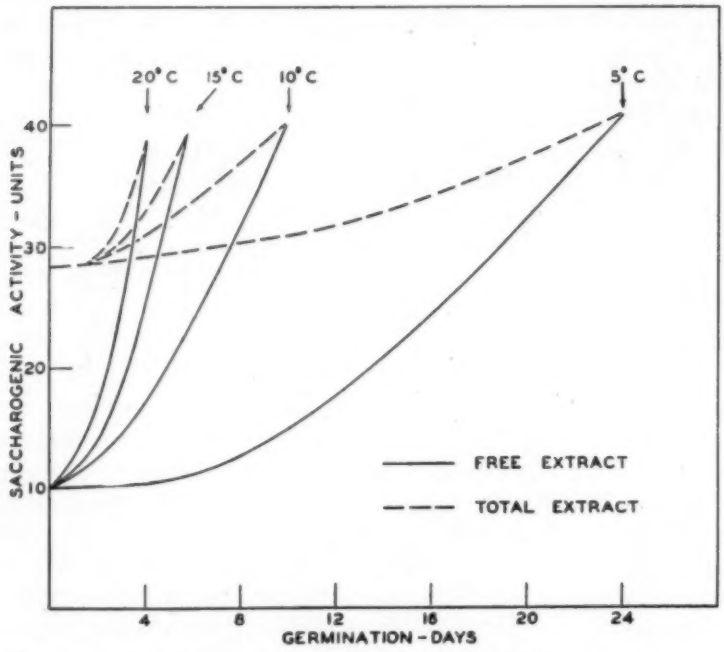

Fig. 1. Saccharogenic activities in the "free" and total extracts of wheat germinated at temperatures of 20°, 15°, 10°, and 5°C.

Beta-amylase activity: The data relative to the development of beta-amylase at the four germination temperatures is shown graphically in Figure 2. The amount of papain-extractable beta-amylase increased slightly in the initial growth stage. This increase was followed by a decline resulting in the activities of the final samples being in all cases below that of the original wheat sample. More striking was the progressive increase in the amounts of free beta-amylase. At the end of the germination periods the free beta-amylase was equal to the total; *i.e.*, there was about three times as much free beta-amylase as originally present. The results of germination at the four temperatures were very similar, about the only difference being that beta-amylase development took place at a decelerated rate as the temperature of germination was lowered.

The interpretation of the small change in activity shown by the total beta-amylase and the rapid increase in "free" beta-amylase to eventually coincide with the total is obvious. As germination progresses the "bound" beta-amylase (that fraction not readily extractable by water) is liberated by the action of the proteolytic enzymes simultaneously formed (Myrbäck and Myrbäck, 1933; Hills and Bailey, 1938a) until a point is reached at which none remains in the bound state.

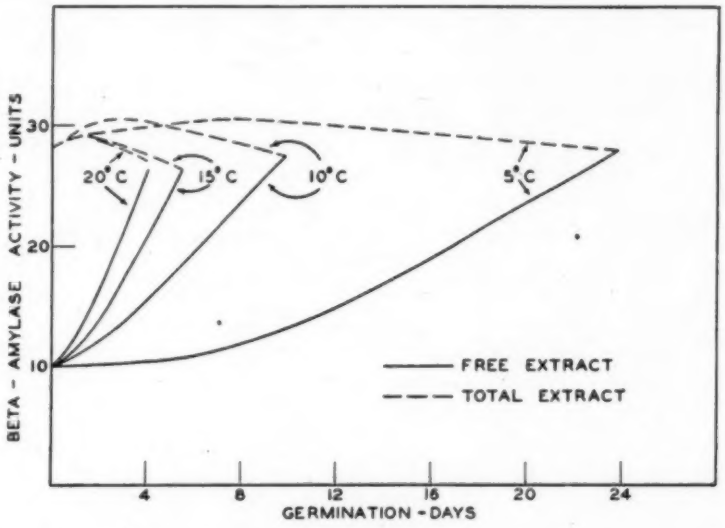

Fig. 2. Beta-amylase activities in the "free" and total extracts of wheat germinated at temperatures of 20°, 15°, 10°, and 5°C.

No other data are available which record accurate measurements of beta-amylase. However, acid inactivation of the alpha component has been used by several workers in an attempt to isolate the action due to beta-amylase. Using this technique Lüers and Rümmler (1935) and Ugrümow (1935), working respectively with barley and wheat, reported that the initial increase of "beta-amylase" was not maintained in the later stages of seedling growth. The results as recorded in Figure 2 do not indicate such a leveling off in activity for the free beta-amylase but conform more closely with the data reported by Ohlsson and co-workers (1932–1938) for germinating cereals.

Alpha-amylase activity: The development of alpha-dextrinogenic activity in germinating wheat is illustrated by the data recorded in Figure 3. Only the results found for the activities of the total extracts are shown. The data of Table I indicate that the developments of "free" and total alpha-amylase are essentially parallel phenomena.

Too, the extraction techniques used resulted in the free extracts' exhibiting nearly as much alpha-amylase activity as the total; the three-hour period used for the water extractions apparently permitted sufficient proteolytic activity to liberate most of the "bound" alpha-amylase. Shorter extractions were used later with a few of the samples and a much wider spread between water- and papain-extractable alpha-amylase was found.

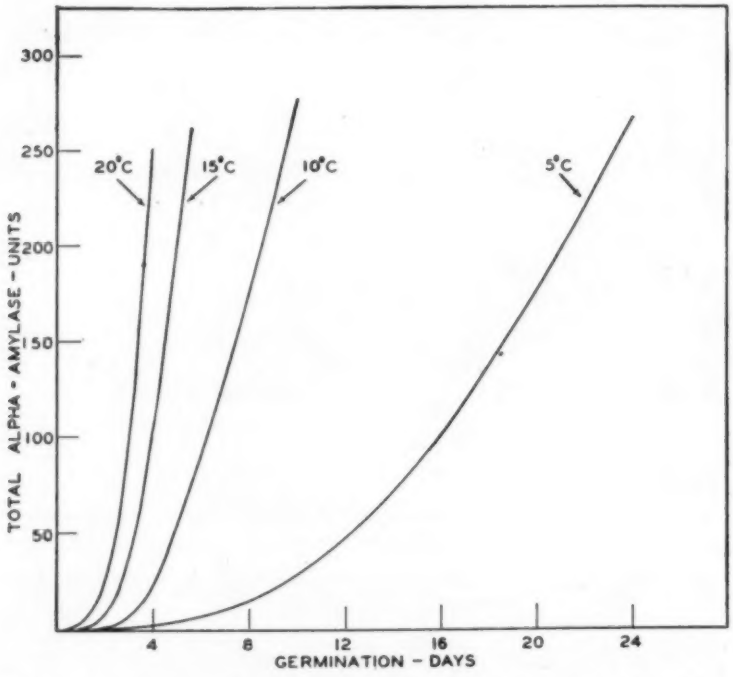

Fig. 3. Alpha-amylase activity in the total (papain) extracts of wheat germinated at temperatures of 20°, 15°, 10°, and 5°C.

The curves of Figure 3 are somewhat similar to those resulting from the free beta-amylase data recorded in Figure 2. After an initial slow increase in alpha-amylase the rate of development is greatly accelerated. Most workers have reported that alpha-amylase does not appear until after a period of germination ranging from one to several days. Application of the method sensitive to very small amounts of alpha-amylase indicates that such development progresses from the very beginning of germination (Figure 3 and Table I). Chrzaszcz and Janicki (1933) also were able to detect a steady increase in dextrinizing enzyme through the first stages of the development of barley seedlings.

So-called alpha-amylase methods used previously in studies of germination have not been specific for the alpha component (Sandstedt,

Kneen, and Blish, 1939). However, the development of "liquefying activity," shown by Kneen, Beckord, and Sandstedt (1941) to be essentially synonymous with alpha-amylase activity, has been studied by Effront (1905), Lüers and Rümmler (1933), and Chrzaszcz and Janicki (1933). Too, the development of that portion of the alpha-dextrinogenic activity remaining after heat inactivation of the beta component has been studied by Ohlsson and co-workers (1932–1938), Ugrümow (1935), and Lüers and Rümmler (1935). The progressive increase in alpha-amylase throughout seedling development shown by the data as recorded in Figure 3 is in fair agreement with the findings of these authors.

The nature of the increase of alpha-amylase activity resulting from papain extraction of barley malt has been attributed by Hills and Bailey (1938a) to an activation of the alpha component already present

TABLE III

EFFECT OF PEPTONE, BOTH DURING AND FOLLOWING EXTRACTION, ON ALPHA-AMYLASE ACTIVITY

Extract and treatment	Alpha-amylase activity
	units
Papain extract	66.7
Peptone extract—"Difco"	56.1
Peptone extract—"Witte"	57.1
Water extract	50.0
Water extract + "Difco" peptone	50.0
Water extract + "Witte" peptone	50.0

in solution rather than a liberation of "bound" enzyme. This conclusion was based chiefly on the finding that peptone stimulated the dextrinizing activity of a solution of alpha-amylase. To investigate this hypothesis several different types of extraction of a malt were made as well as attempts to activate the alpha-amylase in a "free" extract. All extractions were for 30 minutes and with the same ratio of malt to extraction liquid (1 g malt plus 100 ml of a solution containing 1 mg calcium acetate per 1 ml. of water) Two commercial peptones were used, "Difco" and "Witte." In the "peptone extracts" peptone was added to the malt meal before extraction at the rate of 50 mg per gram of malt. For the "papain extract" papain was added to the dry meal at the rate of 100 mg per gram of malt. Where peptone was added to the "free" extract concentrations were adjusted to the same level as used for extraction. The results are shown in Table III.

The data of Table III indicate that the two peptones were approximately alike in their effects. Their presence during extraction resulted in an extract of somewhat greater alpha-amylase activity than the

"free" but considerably less than that of the papain extract. This lends support to the finding of Chrzaszcz and Janicki (1933) that peptone extracts had higher amylase activity than water extracts. However, the data indicate that this increase in alpha-amylase activity by peptone extraction is a liberation of alpha-amylase and not an activation of the enzyme already present in solution. Neither brand of peptone had any effect on alpha-amylase activity when added to the water extract. The present data indicate then that, as with beta-

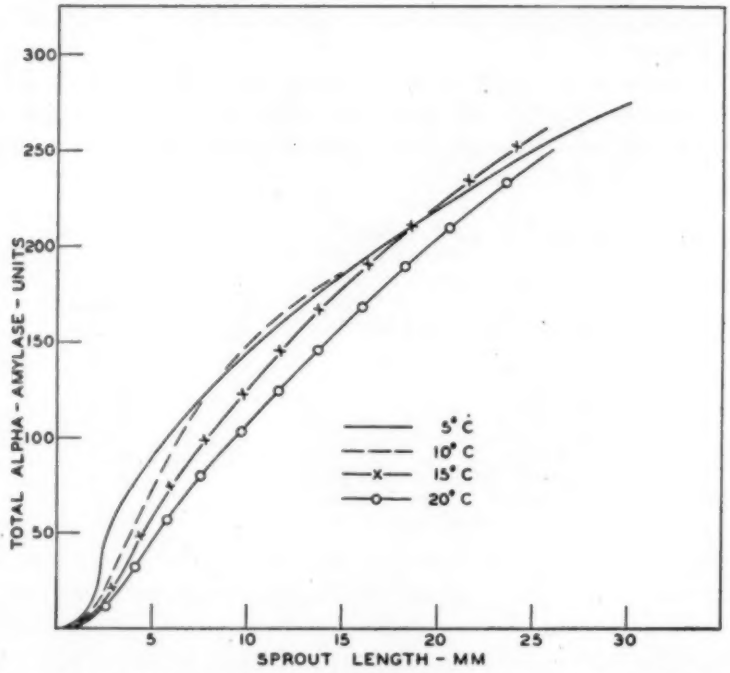

Fig. 4. Relationship between alpha-amylase activity and sprout length of wheat germinated at temperatures of 20°, 15°, 10°, and 5°C.

amylase activity, the action of papain is to liberate a fraction of alpha-amylase that is resistant to the solvent action of water.

Sprout elongation and alpha-amylase development: It may be seen from Table I that the appearance of sprouts is preceded by a considerable development of alpha-amylase. As growth progresses this enzymic development roughly parallels sprout elongation. This is shown by the data as illustrated in Figure 4. It is also apparent that there is a temperature relationship. Particularly in the early stages of seedling development, the lower the germination temperature the higher the amount of active alpha-amylase per millimeter of sprout length. For example the processes leading to the development of 5 mm of sprout

length resulted in twice as much alpha-amylase at 5°C as at 20°C. It should be remembered of course that a much greater time was required for the production of both sprouts and enzyme at the lower temperature. In the later stages of germination the temperature effect was minimized and seedlings with equal sprout lengths were approximately equal in alpha-amylase activity.

Absolute changes in enzyme content: It has been pointed out above that very little loss in total dry weight was caused by germination at either 15° or 10°C. However, the weight loss during the 4 days at 20°C was considerable. Amylase activity was calculated per gram of

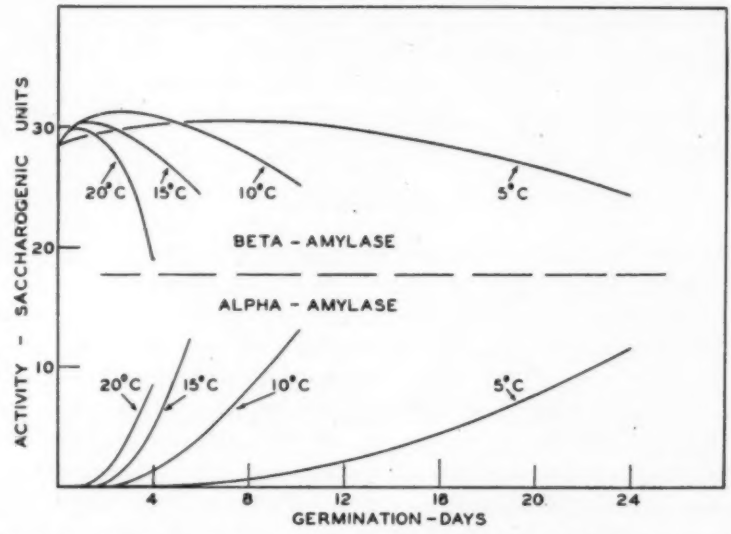

Fig. 5. Absolute changes in the contents of total beta- and alpha-amylase in wheat germinated at 20°, 15°, 10°, and 5°C. (Activities of both components are calculated in terms of saccharifying power.)

dry germinated grain and as such is indicative of an absolute gain or loss only in cases where the total dry weight of the germinated sample was identical with that of the original ungerminated wheat sample. Accordingly the data for the total alpha- and beta-amylase in the seedlings were recalculated on the basis of the dry weight of the original grain. Such values should then be indicative of absolute changes in the amounts of these enzymes.

The data illustrating absolute changes are shown in Figure 5. Alpha-amylase activity, as well as beta-amylase activity, is expressed in terms of saccharogenic units. These "alpha-saccharogenic" units were calculated by subtracting beta-saccharogenic units from malt-saccharogenic units. By this means the activities of the two components were placed on a more comparable basis. As in previous

figures the curves representing total beta-amylase development do not follow the erratic results obtained in the early stages of germination but indicate the general trends.

As diagramed in Figure 5 the data show that there was an apparent increase in the amount of total beta-amylase during the initial stages of germination, followed by a pronounced and progressive decrease. Simultaneously with this decrease in beta-amylase, there was a progressive increase in the total amount of alpha-amylase present. Changes were similar at all four germination temperatures, differences being essentially a matter of rapidity.

It would appear that there must be some relationship between the loss of beta-amylase and the appearance of and increase in content of alpha-amylase. Certainly the relationship between these two changes is too close to permit the conclusion that two *unrelated* processes occur simultaneously: a loss, or inhibition, of beta-amylase and a synthesis, or activation, of alpha-amylase.

Discussion

The studies reported above possibly have little practical application in the production of malt destined for use in the brewing industry. The germination conditions differed greatly from those customarily used in malt production. Moreover, the amylase content of brewer's malt is only one of the many factors influencing malt "quality." However, amylase activity (Geddes, Hildebrand, and Anderson, 1941), and more specifically alpha-amylase activity (Kneen and Sandstedt, 1941b), is the major and probably the only factor of any great significance in the evaluation of a malt for flour supplementation.

In a separate communication (Kneen and Sandstedt, 1941b) it is suggested that malts for flour supplementation be purchased on the basis of their alpha-amylase activity. This necessitates production and marketing of such malts on a similar basis, *i.e.*, on the basis of alpha-amylase content. In other words problems involved in the production and use of malt supplement are essentially those pertaining to the most efficient production and use of malt alpha-amylase. Geddes, Hildebrand, and Anderson (1941) have demonstrated that modification of standard malting procedure may be desirable for the production of such malts. The present study was designed to provide additional information regarding the relationship of certain germination conditions to the development of amylases.

The data indicate that, with the conditions used, the most efficient production of alpha-amylase was at the intermediate temperatures of 15° and 10°C. At these temperatures very high levels of alpha-amylase coincided with a minimum weight loss. A higher temperature (20°C)

resulted in a very rapid production of the enzyme but involved considerable weight loss. Further studies to determine the conditions most suited to the practical production of malt alpha-amylase are essential.

Summary

Hard red winter wheat was germinated at four temperatures and examined periodically to investigate changes in green and dry weight, sprout length, and the saccharogenic, beta-amylase, and alpha-amylase activities of the "free" and total (papain) extracts. Changes taking place at the different temperatures were quite similar, differing essentially only in the rapidity of change. At the germination temperatures of 20°, 15°, 10°, and 5°C approximately 4, 6, 10, and 24 days respectively were required to produce equal levels of sprout length and amylase activity.

Green weight of the developing seedlings increased steadily throughout the germination period. On the other hand total dry weight showed some decrease, this decrease being most pronounced at the 20°C germination temperature.

Both free and total saccharogenic activity (per gram of dry sample) increased progressively throughout germination, the free extract becoming equal in activity to the total after seedling development had progressed to the point at which sprout length approximated 30 to 35 mm. Total beta-amylase activity showed a slight increase during the first stages of growth, followed by an effective decrease. Increase in free beta-amylase was progressive throughout, the free and total activities becoming essentially equal in the later stages. Alpha-amylase activity, both free and total, increased steadily with seedling growth, in this respect essentially paralleling increase in sprout length.

When calculations were based on the original dry weight of the sample it was found that there was a very considerable decrease in the amount of total beta-amylase through the later stages of growth, this decrease being coincident with a similar increase in total alpha-amylase. In addition to changes taking place in the amylolytic activities of the "free" extracts, in part attributable to the action of the proteolytic enzymes developing simultaneously, there was then an apparent loss or inhibition of beta-amylase and a synthesis or activation of alpha-amylase. The suggestion is advanced that these changes may be closely related.

The significance of the data as applied to the production of malt for flour supplementation is discussed. Since the alpha-amylase content of a malt apparently is the only factor of any appreciable significance in supplementation it would seem advisable to so regulate malting practice as to bring about the most efficient production of this enzyme.

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AMYLASE AND PROTEASE SYSTEMS OF MALTED WHEAT FLOUR [1]

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(Read at the Annual Meeting, May 1941)

Amylase Activity

Malt products have long been used as baking supplements because of their property of increasing gas production in fermenting doughs. This stimulation of fermentation has been ascribed to the enhanced amylase activity of such malt products. In the instance of barley malt, it was early determined that at least two amylase factors were present. These were designated originally (Kuhn, 1925) as alpha- and beta-amylase because of the property of the first enzyme to form the alpha-glucoside and of the second to produce the beta modification of maltose. Ohlsson (1926) and Nordh and Ohlsson (1932) developed a procedure for separating the two amylase components that was based on the acid-lability of alpha-amylase and the thermolability of beta-amylase.

[1] Paper No. 29, Journal Series, General Mills, Inc., Research Laboratories.

These components were found to differ markedly in the manner of their action on the starch substrate, which, in this case, was a gelatinized starch paste. It was found that alpha-amylase apparently ruptured the starch molecule at more or less central linkages, producing degradation products of relatively high molecular weight and bringing about concomitant rapid decrease in viscosity and loss of the characteristic blue color with iodine and the property of being precipitated by 60% aqueous alcohol. Each of the three last-mentioned characteristics has been utilized by various investigators as a means of following alpha-amylase activity.

Beta-amylase was found characteristically to act on starch so as to form hydrolysis products predominantly of low molecular weight. In the presence of beta-amylase, it has been found that there is a relatively rapid increase in reducing power, and the blue coloration with iodine characteristic of undegraded starch persists for a much longer period than in the instance of the action of alpha-amylase. An excellent review of these and other considerations involving the amylase systems of cereals has been given by Hanes (1937).

Applying Ohlsson's technique for amylase separation, Andrews and Bailey (1934) showed that in flours milled from normal wheat, maltose production is due primarily to the action of beta-amylase. These authors further demonstrated that during germination, alpha-amylase is produced or activated, and malted wheat flours are accordingly a good source of this factor.

More recent studies on the amylase systems of malted wheat flour have been carried out largely by Blish, Sandstedt, and their co-workers. In 1937, Blish, Sandstedt, and Mecham confirmed the findings of Andrews and Bailey and advanced evidence indicating the presence of a third factor, predominantly occurring in malted wheat, which had the property of attacking raw starch. Fractionation studies with ammonium sulfate, using both boiled and raw starches as substrates, showed that this "raw starch factor" and alpha-amylase were not identical although the two were found to be very similar in their properties.

It is now believed that the efficacy of malted wheat flour in increasing gas production in fermenting doughs is due to hydrolysis of high-molecular-weight starch particles by alpha-amylase or the raw-starch factor with the formation of dextrins of relatively low molecular weight. These dextrins are in turn hydrolyzed by beta-amylase, of which there is apparently an excess in all flours, thus providing an adequate supply of maltose for yeast fermentation. The work of Blish, Sandstedt, and Mecham has also suggested the presence in malted wheat flour of a nonenzymic inhibitor and a nonenzymic "activator."

Protease Activity

An exhaustive study of the proteolytic activity of malted wheat flours has been made by Mounfield (1936a, 1936b, 1938). In these studies Mounfield, using edestin as a substrate and following the extent of proteolysis by determination of the increase in free amino or carboxyl groups by the Sörensen formaldehyde titration, found that sprouted wheat contained an enzyme capable of hydrolyzing the whole protein at an optimum pH of 4.1 at 40°. He also demonstrated the presence of a dipeptidase capable of hydrolyzing leucylglycine and glycylglycine at an optimum pH of 7.5. Both enzymes were found to be spontaneously inactivated on standing in aqueous solution at room temperature.

TABLE I

PROTEASE ACTIVITY OF MALTED AND UNMALTED WHEAT AND WHEAT FLOUR

Material	Proteolytic activity	
	Unactivated	Activated
DATA OF BALLS AND HALE (1936)		
Whole wheat flour (*a*)	1.95	2.40 [1]
Whole wheat flour (*b*)	0.70	1.15 [2]
Wheat malt	3.00	3.15 [2]
White flour	0.45	0.75 [2]
DATA OF HILDEBRAND (1939)		
Patent flour, *A* mix	0.3	
1st clear, *A* mix	0.9	
2nd clear, *A* mix	3.6	
Patent flour, *B* mix	0.6	
1st clear, *B* mix	1.2	
2nd clear, *B* mix	4.8	
Malt flour (*a*)	2.0	
Malt flour (*b*)	6.2	
Ground wheat malt	3.9	

[1] Glutathione activation.
[2] Cysteine activation.

It was found that during the process of germination the proteinase activity of wheat increased approximately six times in four days and ten times in seven days, that the spontaneous inactivation found during storage in aqueous suspension could be materially reduced by adding glycerol to a 45% concentration, and that both enzymes were activated by cyanide. In the last respect these findings are in agreement with the work of Balls and Hale (1936), who have shown that the proteases of wheat are activated by reducing substances.

Mounfield (1938) demonstrated that results obtained with edestin as the substrate could not be quantitatively applied where either gluten or a mixture of glutenin and gliadin were used. In sharp contrast to

the effect previously noted by the same author and to the studies of Balls and Hale, no activating effect of cyanide could be found when gluten was used as substrate.

Quantitative data on the proteolytic activity of malted wheat flours are relatively meager. In Table I are shown typical results taken from the studies of Balls and Hale (1936) and Hildebrand (1939). Since the data were obtained by two different methods of determination, they may not be inter-compared, but they will serve to show the relative activity of malted wheat and malt flours compared to that of unmalted wheat and flour. Balls and Hale, measuring the increase in amino groups by alcoholic titration, found that wheat malt had approximately seven times the protease activity of patent flour and from two to three times that of whole-wheat flour. The values given in the lower section of Table I, obtained by the rate-of-gelation procedure

TABLE II

PROTEINASE ACTIVITY OF MALTED AND UNMALTED WHEAT FLOURS

Sample	Proteinase activity (as mg nonprotein N/100 g)		
	No yeast	3% yeast	3% yeast+ octyl alcohol
Patent flour *A*	117	94	(98)
Patent flour *B*	48	70	(28)
Patent flour *C*	31	50	(26)
Malt flour *A*	257	310	348
Malt flour *B*	233	276	301
Malt flour *C*	317	336	378

of Landis and Frey (1938), indicate that malt flour is from three to twenty times as active as patent flour but may be lower in activity than a second clear flour produced from unmalted wheat.

Similar comparisons may be made from the data in Table II, first column. These values, obtained by the Ayre and Anderson (1939) trichloracetic acid precipitation method, indicate that malt flours are from 2.5 to 5 times as active as patent flours. The data shown are typical of a large number of determinations carried out on flours from both unmalted and malted wheats.

In Table II is also shown the effect of the presence of yeast on proteolytic activity. When 3% of yeast based on the weight of flour was included in the autolytic digests, there was a fairly regular increase in production of nonprotein nitrogen, as shown by the data in the second column. These values, however, are undoubtedly too low because of the utilization of some nonprotein nitrogen by the respiring yeast. Freilich and Frey (1942) suggested the use of octyl alcohol to

inhibit yeast respiration. The data in the third column of Table II show the results obtained in the presence of both yeast and octyl alcohol. In the instance of malted wheat flours, it is evident that there is an apparent further increase in the production of nonprotein nitrogen, and it is believed that these last figures are a true measure of proteolytic activity of such material in fermenting doughs. The data obtained with patent flour under these conditions, however, are open to question. It was exceedingly difficult to obtain reproducible results under these conditions, and there seems to be no adequate explanation for the apparent large decrease in nonprotein nitrogen here noted.

Effect of Malted Wheat Flour on Dough Softening

It has been shown repeatedly that the addition of excessive amounts of malted wheat flour to doughs causes increased mobility and, with extended fermentation periods, may give rise to excessive stickiness. Early workers were of the belief that these effects were due entirely to protein hydrolysis, but in 1933 Kosmin advanced the theory that the production of sticky doughs and moist crumb was due to liquefaction and dextrinization of starch. Brief experiments led to her belief that the hydrolytic changes in gluten structure were not of sufficient magnitude to account for the effects noted. This theory has received increasing support from other investigators in succeeding years. Thus Read and Haas found that malted wheat flours had a relatively low proteolytic activity compared to that of other proteases and therefore concluded that the sticky quality of doughs containing high dosages of malted wheat flour must be attributed to alpha-amylase. In 1936, the same authors treated aqueous extracts of various proteolytic agents with safranine according to the technique proposed by Marston (1923) in order to remove proteases. They found that malt preparations thus treated and presumably, therefore, free from proteases were still able to cause stickiness in doughs.

Bohn and Bailey (1937) in studying changes in physical properties of doughs found that the addition of malted wheat flour appreciably lowered stress readings. Although doughs prepared with 1% and 2% malted wheat flour were found to be more sticky and mellow than fermented doughs with 0.5% malt, differences in physical behavior were not detectable by either stress or five-minute farinograph measurements. These authors state: "It has not been proven that the softening effect is entirely due to the papainase enzyme present in flour. It is possible that other enzymes, proteolytic, diastatic, or otherwise, may also be partly responsible for the mellowing effect. It is difficult to obtain pure proteolytic enzymes free from amylases and *vice versa*, and thus the problem as to what enzyme or enzymes

cause the softening effect upon the gluten or dough system remains unsolved."

Munz and Bailey (1937) obtained alpha- and beta-amylase preparations from malted wheat flours by the Ohlsson technique. The protease content of these preparations was reduced by precipitation with safranine. Using the farinograph to follow changes in dough consistency, it was found that increase in mobility was obtained only in preparations containing alpha-amylase. When this enzyme was inactivated by acid treatment, extracts exerted no significant effect on dough properties.

Sandstedt, Jolitz, and Blish (1939) concluded that stickiness in doughs is associated with the presence of amylodextrins produced by the action of alpha-amylase, lending further support to the belief that alpha-amylase or some factor associated with it in malted wheat flour is responsible for stickiness.

Geddes, Hildebrand, and Anderson (1941), in a study of the effect of malting conditions on the properties of malted wheat flour, utilized a series of experimentally produced malted wheat flours having a fairly wide range of proteolytic activity. However, when such flours were added to a common untreated base flour in amounts sufficient to give constant and adequate gas production, the amount of proteinase activity contributed by the malted wheat flours was a very small fraction of the total activity in the blends. In no case could as much as 5% of the total activity be ascribed to the malted wheat flour. However, in the experiments described above, the experimental malting conditions were not varied as widely as possible, and, at the time the proteolytic activity determinations were carried out, the malted wheats had been in storage for an extended period. While it was believed that such storage would not affect relative results, it does cast some doubt on the reliability of the absolute values found.

In connection with another problem, the authors have had available a series of 48 experimentally produced malted wheat flours exhibiting a wide range in proteinase activity. In order to check the results reported by Geddes, Hildebrand, and Anderson, the amount of each of these flours required to give a constant and adequate gas production was determined and a series of blends comprising the required amount of each malted wheat flour, with a common untreated base flour, was prepared. Gas production determinations were made by the method described by Sherwood, Hildebrand, and McClellan (1940), and it was found that the blends gave a mean gas production of 147 ml of CO_2 from the second to the fifth hour of fermentation, with a standard deviation of ± 2.0 ml. Calculation of the proteinase activity contributed by the malted wheat flours showed that this amount ranged

from 0.08 to 4.0 mg of nonprotein nitrogen per 100 g, whereas the base flour employed (patent flour *A* in Table II) contributed 117 mg per 100 g. It is obvious, therefore, that even under the most unfavorable circumstances the amount of proteinase activity that can be ascribed to added malted wheat flour is an extremely low percentage of the total.

In spite of the apparent low proteolytic activity due to malted wheat flour in these blends, it was deemed desirable to ascertain whether or not the variation in such proteolytic activity would exert an appreciable effect on dough-handling properties and/or baking characteristics. To this end, the 48 blends described above were baked in duplicate in random order by the basic A.A.C.C. procedure with the exceptions that a four-hour fermentation period was employed in order to accentuate any differences that might be found, and 5% sugar was included in the formula to insure adequate gas production under this extended fermentation period. No evidence whatever could be found of significant variations in loaf volume, crumb, or crust characteristics or dough-handling properties among the doughs thus baked. Accordingly, it must be concluded that, while the proteolytic activity contributed by the malted wheat flours under these conditions varied from approximately 0.1 to 4 mg of nonprotein nitrogen, such variation was totally without effect on baking properties.

The experiments described above have been supplemented with other studies designed to ascertain the factor responsible for dough softening in the presence of excessive treatments of malted wheat flour. Malted wheat flour of relatively high amylase activity and low proteinase activity was extracted and the extract treated with safranine according to the technique described by Marston (1923). The latter author states that separation of proteinase is complete only in the absence of protein degradation products. The extract was accordingly tested for amylase and proteinase activity, both before and after treatment with safranine. It was found that the extract had the same capacity for stimulating gas production as did an equivalent weight of the original malted wheat flour, and accordingly it was assumed that quantitative extraction of alpha-amylase and/or the raw starch factor described by Blish, Sandstedt, and Mecham had been attained.

In order to determine the effect of safranine treatment on the proteolytic activity of this extract, equal portions of the extract before and after safranine treatment were allowed to act on casein for one- and three-hour periods. The extent of nonprotein nitrogen production was determined by the usual technique, and it was found that treatment with safranine had reduced proteolytic activity by approximately one-third. On this basis, the proteolytic activity of this extract when

acting on flour protein was also assumed to have been reduced to this extent.

To 300-g portions of an untreated base flour were added increasing amounts of this extract, 3% yeast, and sufficient water to give the proper mobility. The doughs were mixed in the farinograph, removed, and allowed to ferment for four hours at 30°. At the end of this period, 450-g aliquots of each dough were scaled and remixed. It was noted that with increasing increments of malted wheat flour, there was a progressive increase in stickiness of the doughs and a progressively greater increase in mobility between the original doughs and those remixed after four hours of fermentation. The results obtained are given in Table III. The addition of safranine precipitate equivalent to 5% of malted wheat flour did not give a significant alteration in dough consistency or stickiness.

TABLE III

EFFECT OF SAFRANINE-TREATED EXTRACT OF MALTED WHEAT FLOUR ON DOUGH MOBILITY

Extract added, as malt flour equivalent	Proteinase activity due to		Fraction of total PA due to extract	Increase in mobility in 4 hrs at 30°
	Extract	Base flour		
%	*mg nonprotein N*		%	*Brabender units*
0	0	360	—	175
1	7.4	360	2.0	210
2	14.8	360	3.9	200
5	37.0	360	9.3	235
10	74.0	360	17.1	300
Proteinase ppt. equiv. to 5% malted wheat flour	55.2	360	13.3	185

The results described above tend to substantiate the belief previously advanced that the increase in stickiness and in dough mobility noted with excessive treatment of malted wheat flour cannot be ascribed to proteolytic activity of such flours but are due rather to excessive alpha-amylase activity.

Summary

Brief reviews of the present state of our knowledge of the amylase and protease systems of malted wheat flours have been given.

Evidence is advanced to support the belief that increase in dough mobility and stickiness noted with the addition of excessive dosages of malted wheat flour is to be ascribed to alpha-amylase activity contributed by such flours rather than to proteinase activity.

Acknowledgment

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NUTRITIVE VALUE OF THE PROTEIN AND MINERAL CONSTITUENTS OF RICE VARIETIES

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Extensive feeding experiments with pigeons, on the antineuritic values of several samples of Indian rice, both raw and parboiled, have been carried out by McCarrison and Norris (1924). Spruyt (1930) has reported that his colorimetric method of estimating vitamin B_1 in rice samples gives results in complete accord with those obtained by feeding the samples of rice to rice birds (*Munia* birds). Aykroyd (1932), using the method of biological assay of Chick and Roscoe (1929), has investigated the comparative vitamin B_1 contents of raw and parboiled rices, polished to different degrees. The biological value of the proteins of rice has been determined by a few workers (McCollum and Simmonds, 1917; Osborne and Mendell, 1918; Mitchell and Villegas, 1923; Mitchell and Carman, 1924, 1926; Morgan, 1931; Swaminathan, 1937; Basu and Basak, 1937; and others) and it has been recorded that rice, in spite of its somewhat low protein content, has a biological value higher than that of most other cereals, including wheat.

Apart from these studies, very little attention seems to have been directed towards following comparative rates of growth, in young rats or pigeons, when fed different varieties of rice as the sole source of proteins and minerals in an otherwise adequate diet. It was therefore decided to investigate the possibility of such varietal differences.

Experimental

Series I: Six varieties of rice, Adt 3, Co 9, Co 10, Adt 7, GEB 24, and Adt 11, were chosen for this experiment. These showed somewhat similar tendencies in regard to protein and mineral variations. The powdered samples of the (unpolished) rices were steamed in the dry state at 120°C. in an autoclave for 2½ hours on two successive days. This period was sufficient to inactivate the antineuritic vitamin present in the samples.

Composition of rice varieties: Table I gives the protein and mineral contents of the varieties of rice (shelled, unpolished) used in this and following experiments.[1] The methods of analysis were those of the A.O.A.C. (1935).

[1] These samples were obtained through the kind courtesy of the Government Paddy Specialist, Coimbatore, Madras.

Technique of feeding: It was found that cooking the rice grains before feeding did not supply a good food, as the material tended to set into a hard horny mass on partial drying, thus making it very difficult to be used by the rats. Besides, a good portion of the feed was scattered by the animals when fed in this form. Hence, in these experiments, the rice was powdered and with the other supplements, made into a sort of cake. This was done by making a fairly thick paste of the flour with water and pouring it on a hot plate well smeared with arachis oil. Sufficient quantity of food in the form of cake was given to the different rats.

To the rice samples, autoclaved in the dry powdered condition at 120°C., vitamin B_1 was supplied from an external source.

TABLE I

COMPOSITION OF RICE SAMPLES (OVEN-DRY BASIS)

Variety	Protein	Total mineral	P_2O_5	CaO
	%	%	%	%
Adt 6	8.67	1.85	1.28	0.10
Adt 3	10.47	1.62	0.82	0.12
Co 9	9.59	1.76	0.73	0.10
Co 10	7.61	1.65	0.79	0.06
Adt 7	7.26	1.70	0.56	0.13
GEB 24	6.97	1.66	0.54	0.07
Adt 11	6.59	1.46	0.70	0.09

Groups of four to six young rats,[2] about 30 days old, were given sufficient quantities of the rice powders in the form of cakes, together with the same supplements of cod-liver oil (0.05 g. daily) and marmite extract (2 cc. of a 5% extract, daily). The latter were uniformly smeared on the cakes so as to keep their proportions in the different diets more or less constant.

Growth charts were maintained for the different rats in each of the six groups for a period of seven weeks. At about this period the rate of growth began to decline in all the groups; in a few cases, the animals did not appear quite healthy and their fur dropped gradually. Since the diets consisted mainly of rice, the protein level of the food was only between 6.6% and 10.5%; obviously, this was not optimal for growth over prolonged periods. Hence, a small supplement of casein (3% by weight of the diet) was now given and the experiments continued for another seven weeks. It was not assumed that a supplement of this order would introduce additional variables in the experiment. The average growth

[2] Sometimes unequal numbers of animals had to be used for the reason that sufficient young rats were not always available, especially when 20 or more rats were required at one time. In other experiments where the same number of rats were used for each group to start with, there were occasional deaths in the middle of the experiment. It has however been recorded in the text of the paper wherever such deaths occurred.

curves for the different groups are given in Figure 1, while the growth rates are presented in Table II.

The results have been examined statistically by Fisher's analysis of variance (1935). The weekly average increase in weight for the different groups ranged approximately between 4 and 7 g. with the varieties. The average rate for the period before the casein supplement was given was invariably more than for the entire 14 weeks. This may be due to the fact that the rate of growth in young rats, even with inadequate diets, is greater during the first few weeks and falls down with

TABLE II

EFFECTS OF VARIETAL FACTORS ON AVERAGE WEEKLY GROWTH RATE (SERIES I)

Variety of rice	Number of rats used	Average weekly increase in weight in g. during		Remarks
		7 weeks	14 weeks	
Adt 3	5	7.49	6.00	One rat died on 69th day.
Co 9	4	6.96	5.67	One rat died on 74th day.
Co 10	5	5.97	5.00	One rat died on 61st day.
Adt 7	4	4.75	4.29	One weighed maximum 86 g. (stunted) and died on 88th day.
GEB 24	5	4.88	4.36	—
Adt 11	6	4.10	3.79	One rat died on 58th day and another on 79th day.
Standard error per sample:		0.27	0.26	
Significance:				
Without casein		Adt 3	Co 9	Co 10 Adt 7 GEB 24 Adt 11
With casein		Adt 3	Co 9	Co 10 Adt 7 GEB 24 Adt 11

age. It may be noted however that, between the varieties, the order in which growth rates decreased significantly was more or less the same with and without the casein supplement.

In another report (Sreenivasan and Sadasivan, 1941), the comparative nutritive values of the protein and mineral constituents of three varieties of rice grown under (a) dry, (b) swamp, broadcast, and (c) transplanted, swamp conditions were studied by the same technique as above. The effect on growth of the protein and mineral contents of these samples and the six varieties above (Table II) were examined together and it was found that the average growth rates due to the different rices depended upon their contents of proteins (x_1), phosphorus compounds (x_2), and minerals excluding phosphate (x_3). Each of these was correlated with the growth rate (y) during the precasein period of seven weeks by means of a regression equation:

$$y = 0.42x_1 + 7.34x_2 + 3.43x_3.$$

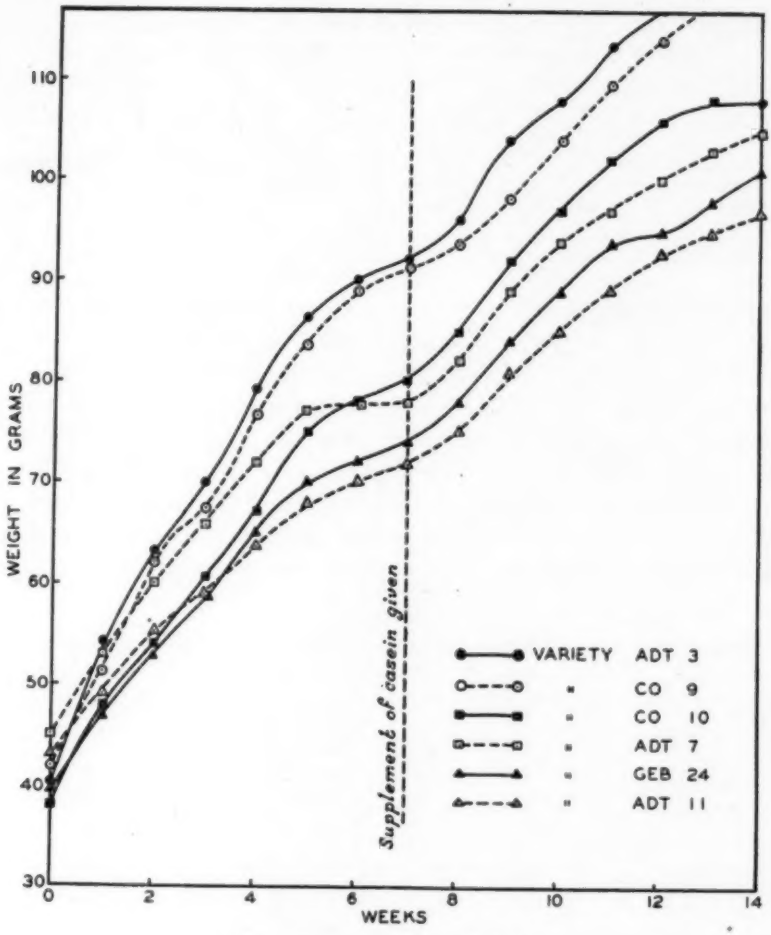

Fig. 1. Average growth rate, Series I.

The values of partial regression coefficients were:

	S.E.	*t*	*n*	*P*
$b_1 = 0.42 \pm 0.05$		8.4	61	0.01
$b_2 = 7.34 \pm 1.28$		5.7	61	0.01
$b_3 = 3.43 \pm 0.57$		6.0	61	0.01

The value of the multiple correlation coefficient R was $+ .9232$ and was significant on the 1% level (Table III).

Series II: The protein levels of the diets used in the above series were somewhat low as compared to the normal requirements of rats. Besides, the animals did not receive any supplements of minerals other than what was contained in the rice varieties themselves. Since it was observed that growth was better correlated with phosphorus than with

TABLE III

TEST OF SIGNIFICANCE OF MULTIPLE CORRELATION COEFFICIENT

Variance due to	Degrees of freedom	Sums of squares	Mean square	Ratio of variances	Remarks
Regression	3	80.5800	26.8600	117.1902 [1]	Significant
Deviation	61	13.9809	0.2292	—	—
Total	64	94.5609	—	—	—

[1] Significant on 1% level.

the total minerals, it was considered desirable to study the effect of supplementing the rice diets with phosphate-free salt mixture on growth. Accordingly, in the following series, the nutritive values of two rice samples (GEB 24 and Co 9) were compared exactly as above, using groups of five animals for each rice. But the animals received in addition a daily supplement of 5% of the rice as a phosphate-free salt mixture composed of calcium lactate (150 parts), magnesium sulfate (30 parts), ferric citrate (12 parts), sodium chloride (10 parts), and potassium chloride (10 parts).

TABLE IV

EFFECTS OF MINERAL SUPPLEMENTS ON AVERAGE WEEKLY GROWTH RATE (SERIES II)

Variety of rice	Average weekly increase in weight in grams during	
	7 weeks	14 weeks
Co 9	8.43	6.52
GEB 24	7.14	5.60
Standard error per sample	0.30	0.21

Periodic observations on growth rates were made as before and, when the growth rate began to decline at the end of 6 to 8 weeks, casein supplement (3%) was given to the diets and the experiments continued for a further equal period (Table IV and Fig. 2).

The differences in growth rates between the varieties were significant and confirmed the earlier observations in regard to the relationship between growth and nitrogen and phosphorus contents. It may be noted that addition of the salts has increased the growth rate in this series as compared to series I.

Series III:[3] The foregoing experiments have shown that, given adequate supply of the antineuritic and other vitamins, the growth-promoting qualities of different varieties of rice are essentially related

[3] A note embodying the results of this series has since been published by Sreenivasan (1939).

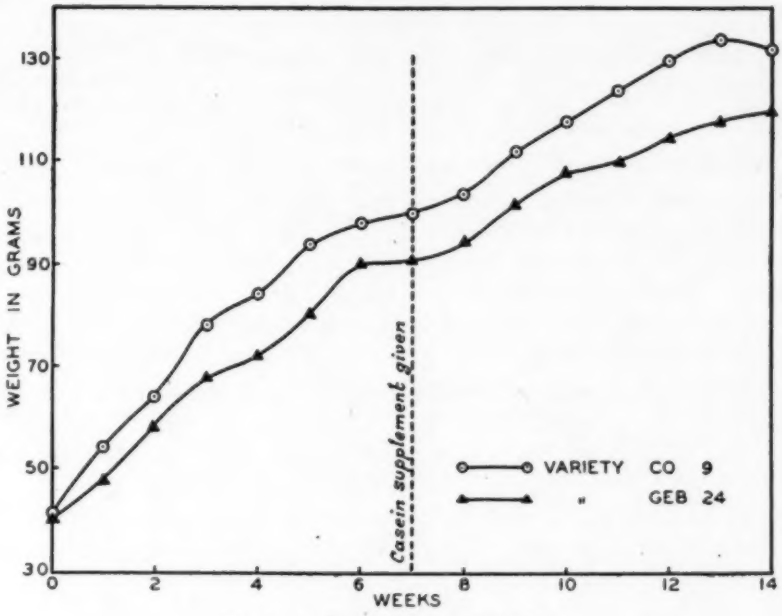

Fig. 2. Average growth rate, Series II.

to their nitrogen and mineral (particularly phosphorus) contents. In the present series, an attempt has been made to compare the growth-promoting qualities of the phosphorus compounds of two of the varieties, Adt 6 and Adt 11. The powdered, autoclaved rices were used for feeding, but in one case (Adt 11) the rice flour was intimately mixed with 2% of a preparation of rice protein (purity 94%) so that the initial protein contents of the two feeds were nearly the same. The diets were then mixed with 5% of phosphate-free salt mixture as in Series II. Groups of four young rats were fed on the two samples thus prepared, together with the same supplements of cod-liver oil and marmite extract. The experiments were continued for a period of six weeks. The weekly growth increases are given in Table V.

TABLE V

AVERAGE WEEKLY GROWTH INCREASES WITH EQUAL PROTEIN LEVELS (SERIES III)

Rice diet	Weekly increase in weight in grams					
	1st week	2nd week	3rd week	4th week	5th week	6th week
Adt 6	13.2	12.6	10.8	8.4	7.0	6.2
Adt 11 + 2% rice protein	9.6	10.4	8.4	6.8	5.4	5.0

Standard error per sample: 0.26

TABLE VI

AVERAGE GROWTH RATES OF PIGEONS WITH DIFFERENT RICE DIETS

Group	Treatments in diet	Protein	P_2O_5	Initial weight	Final weight	Weekly increase in wt.	Remarks
		%	%	g.	g.	g.	
1	Unpolished (Co 9), autoclaved + polishings from Co 9	10.10	0.80	219	255	7.2	—
2	Unpolished (Adt 11), autoclaved + polishings from Co 9	7.82	0.78	222	249	5.4	—
3	Polished (Co 9), autoclaved + polishings from Co 9	8.36	0.64	218	248	6.0	One bird died on 16th day.
4	Polished (Adt 11), autoclaved + polishings from Co 9	7.36	0.52	225	246	4.2	One bird died on 18th day and another on 30th day.
5	Unpolished (Co 9), autoclaved + polishings from Adt 11	8.55	0.89	220	251	6.2	—
6	Unpolished (Adt 11), autoclaved + polishings from Adt 11	6.27	0.87	226	251	5.0	—
7	Polished (Co 9), autoclaved + polishings from Adt 11	6.81	0.73	220	243	4.6	One bird died on 22nd day.
8	Polished (Adt 11), autoclaved + polishings from Adt 11	5.77	0.61	215	233	3.6	—
9	Unpolished (Co 9), control	8.42	0.64	218	250	6.4	—
10	Unpolished (Adt 11), control	5.77	0.61	219	238	3.6	One bird died on 22nd day.
	Standard error per sample: 0.22						
	Significance groups: 1 9 5 3 2 6 7 4 8 10						

The differences between the varieties were highly significant. Thus, in spite of the protein contents having been adjusted to the same level in both the diets, there was a significant difference in growth rates due to the two varieties of rice. This is evidently traceable to the differences in the mineral contents of the grains. Since the diets contained enough supplements of all the minerals except phosphorus, it is reasonable to assume that the better growth induced by Adt 6 was due to its higher phosphorus content which was nearly 1.8 times that of Adt 11.

Experiments with pigeons: Ten groups of young pigeons, four each, were fed for a period of 35 days on two varieties (Co 9 and Adt 11) of rices, unpolished as well as polished, and subjected to different treatments (Table VI). One of the varieties (Co 9) was a coarse-grained red rice, while the other was a medium-grained white rice. Polishing

with each variety was done until the bran was just completely removed from the grain. Where polishings were added to the food, this was done in the proportions in which they existed in the original unpolished rices.

The diets were designed to throw light on the comparative nutritive qualities of the varieties in relation to their mineral, protein, and vitamin B_1 contents. For feeding the birds, the rice grains were cooked in an autoclave with the necessary amounts of water and then mixed with the requisite quantities of bran from red or white rice, together with small doses of cod-liver oil (0.05 g. per bird) and autoclaved marmite extract (2 cc. of 5% extract per bird). Feeding was done *ad libitum*. Growth records were maintained for all the pigeons throughout the period of experiment. Table VI gives the different treatments, the protein and phosphate contents of the diets and the weekly average growth rates of the pigeons in the different groups.

The experiments could not be continued over a longer period as, in most cases, the pigeons began to go down in weight and show signs of ill health. Besides, they showed an aversion to the food. It was therefore concluded that the diets were not palatable and perhaps also did not supply optimal quantities of nutritional ingredients.

The results show that growth, in general, is related to the protein and phosphorus contents; grain colour as such had probably no effect. It appeared that the amounts of vitamin B_1 supplied by the rice polishings, in quantities used in these experiments, were inadeqquate for the birds since head retraction appeared in a few cases after about 30 days. It must be noted however that, since the feeds were predominantly starchy in character, they would, in consequence, require larger amounts of vitamin B_1 for proper metabolism.

Discussion

Young rats fed on a diet in which rice was the only source of protein grew slowly, especially after the first few weeks and showed signs of fur dropping. Growth was stimulated by supplementing the diet with casein. Suzuki, Matsuyama, and Hashimoto (1925) have demonstrated that rats fed on a diet containing rice protein never weighed more than 130 g.; standard growth was obtained only by employing 15% or more of rice protein in the diet. Similar results on the inadequacy of rice as the sole source of protein in diets given to rats have been reported by Hermano (1933), who found that, under such conditions, the animals were stunted and did not weigh more than 104 g. In other experiments carried out by the present author (unpublished data) it has been observed that with diets containing over 90% of rice young rats require

larger feed per unit increase in weight. With pigeons, low-protein diets, although taken in normal quantities during the first few weeks, were not well liked by the birds. They took less food on continued feeding with such diets and showed definite indications of digestive disorders.

Though a rice diet does not continue to maintain growth over prolonged periods, it must yet be borne in mind that, in ordinary practice, rice does not serve as a large proportion of the diet and is generally a part of a good mixed diet, which includes pulses and vegetables. There is no doubt that, under such conditions, rice will have a high nutritive value. Recent work at Bangalore (Ranganathan and Rau, 1938) has shown that the biological value of the proteins of rice in a mixed diet was greatly increased by the addition of calcium. Supplements of skimmed milk or calcium lactate have also been shown to enhance greatly the nutritive value of a rice diet (Wilson, Ahmad, and Mullick, 1936; Aykroyd and Krishnan, 1937). Further work on the effect of other supplements to make rice a complete and well-balanced food needs to be carried out.

The differences in growth rate due to the varieties of rice examined are due to differences in protein and mineral contents. There is a general and widespread belief that early-maturing varieties of rice are inferior to late-maturing ones (Chambliss and Adams, 1915; Wells, Agcaoilli, and Feliciano, 1922; Acton and Chopra, 1925; Basu and Sarkar, 1935; and others). While it is true that long-season crops invariably yield better, there are yet many short-season varieties of rice which contain greater percentages of albuminoid and mineral matter (Sadasivan and Sreenivasan, 1938).

Since the nutritive values of rice varieties are mainly determined by their composition, it would follow that there is great need for improving quality in rice along the lines of enriching its composition by selection of suitable varieties and by proper manuring. Cultural methods and irrigation are important factors in improving not only yield but also the quality of the proteins and minerals as well (Kelley and Thompson, 1910; Wells *et al.*, 1922; Sadasivan and Sreenivasan, 1938).

The increased growth rates observed with addition of phosphate-free salt mixture (Series II) would show that proper supplements of minerals will enhance the nutritive value of rice. Rice contains between 1% and 2% of mineral matter with phosphorus as a major component. The phosphorus occurs mostly as phytin in organic combination (Rather, 1918; McCance and Widdowson, 1935) and, in this form, is not considered as readily available to the animal organism (McCance and Widdowson, 1935; Lowe and Steenbock, 1936). The results of the present experiments (Series III) would show however that with the same level

of protein supply, the variety of rice which is high in phosphorus induces better growth in rats. It cannot therefore be said that the phosphorus in rice is entirely unavailable. It is likely that the culinary conditions or the possible presence of an intestinal phytase in the rat (Patwardhan, 1937) renders at least part of the phosphorus compounds in rice assimilable. The results indicate the need for further work on the ingestion of phosphorus in rice under different conditions, and on the effect of calcium and of the calcium : phosphorus ratio on the availability of the phytin in rice.

Summary

Groups of young albino rats fed different varieties of rice after autoclaving, and with supplements of cod-liver oil and marmite extracts only showed variations in growth rates that were essentially related to protein and mineral contents of the rices. The rates of growth decreased after about two months when the animals attained a weight of 90 to 100 g. Supplements of casein given at this stage resulted in increased growth which, in the different groups, was again proportional to the nitrogen and mineral contents of the rices.

The correlation between rate of growth during seven weeks and the proteins, phosphorus compounds, and minerals other than phosphates has been shown by means of a regression equation.

The weekly average growth rate of young rats fed on rice diets increased when a supplement of phosphate-free salt mixture was given. The differences due to varieties were still of the same order.

In experiments with rice diets containing the same level of protein but with different amounts of phosphorus, it was observed that the growth rate was greater with the variety of rice containing the greater percentage of phosphorus.

Similar results on the relationship between growth and composition of the feeds were obtained in experiments with groups of pigeons fed on two varieties of rice, unpolished as well as polished and with and without the addition of rice polishings.

The significance of these observations in relation to the nutritive values of the protein and mineral constituents of rice varieties and the availability of phytin in rice is discussed.

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NUTRITIVE VALUE OF THE PROTEIN AND MINERAL CONSTITUENTS OF DRY AND WET CULTIVATED RICES

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Perhaps the most important among the requirements of the rice plant is an abundant supply of fresh water, either rain or irrigation, during the major part of the growing season. The crop stands partly submerged in the field for over two-thirds of the growing period. Although the rice plant flourishes best under swampy conditions, there are still certain dry cultivated rices which are grown in certain parts of India and elsewhere. These do not require puddling and prolonged swamping in the same way as the commoner varieties do. Irrigated rice is generally more productive than dry cultivated rice. Even those varieties of rice that are cultivated under dry-land conditions yield more if irrigated.

Dry-land cultivated rices are however popularly believed to be more nutritious than wet-land cultivated rices. According to McCarrison (1928), the conditions of cultivation of rice, especially in regard to water supply during the growth of the crop, are factors of importance in determining the endemicity of beriberi. He has adduced experimental evi-

dence to show that the lower nutritive value of rice grown under wet-land conditions is due in part at least to its lower content of vitamins of the water-soluble B class. If this is generally the case, it would be desirable to extend the method of dry cultivation and, if possible, improve on it so as to combine superior quality with high yield. The problem therefore appeared to merit further study and accordingly the present inquiry was undertaken.

Experimental

Three different varieties of rice, Dirty modan, Black modan, and Katta modan, were grown during April to September, 1936, at the Agricultural Research Station, Pattambi, West Coast, with identical manurial treatments (10 cartloads of cattle manure *plus* 2,000 pounds of wood ash per acre) but under different conditions of cultivation as follows: (1) irrigated, transplanted, (2) irrigated, broadcast, and (3) dry, broadcast. In (1) and (2) the fields were maintained in the puddled condition in

TABLE I

EFFECTS OF DIFFERENT ENVIRONMENTS AND CULTURAL METHODS ON YIELD OF PADDY IN POUNDS PER ACRE

	Dirty modan			Black modan			Katta modan		
	Dry broad-cast	Wet broad-cast	Trans-planted	Dry broad-cast	Wet broad-cast	Trans-planted	Dry broad-cast	Wet broad-cast	Trans-planted
Sowing date	23–4	16–5	16–5	23–4	16–5	16–5	15–5	16–5	16–5
Transplanting date	—	—	10–6	—*	—	10–6	—	—	10–6
Flowering date	10–7	28–7	2–8	10–7	28–7	2–8	5–8	5–8	5–8
Harvesting date	4–8	20–8	4–9	4–8	26–8	4–9	4–9	4–9	4–9
Duration, *days*	103	96	111	103	102	111	110	111	111
Rainfall from sowing to harvest, *in.*	69.6	76.8	79.8	69.6	76.8	79.8	80.9	79.8	79.8
Yield, *lbs. per acre*	824	1,133	1,400	783	716	1,434	740	1,267	1,734

the usual way, but in (1) the seedlings were raised in separate seed beds and transplanted at the end of 26 days, while in (2) the seeds were broadcast in the fields. In (3), the seeds were also broadcast on receipt of pre-monson showers but the soil was maintained in the dry condition, the crop being thus only rain-fed. While the type of land used for the two wet trials was the same, the land used for the dry trial was comparatively poor in fertility, being situated on unbunded hill slopes. This could not be avoided.

Table I gives the yields of paddy at harvest for the different samples, together with other details of the experiment.

It may be observed that, with the exception of Black modan the yield was lower under conditions of dry cultivation than with the puddled soil

(*cf.* Sreenivasan, 1936). In the latter case, transplanted rice invariably gave a higher yield than the same variety broadcasted (*cf.* Howard, 1924). For strict comparison, it would have been better if the sowings had all been done on the same date. As it is, it is difficult to explain the low yield of Black modan from the wet broadcast crop as compared to the dry crop. It must be noted that the yields are only from single plots rather than from replicated experiments.

Chemical composition: The chemical compositions of the shelled, unpolished rices from the different samples were determined according to methods outlined in an earlier publication (Sadasivan and Sreenivasan, 1938). Table II gives the results.

TABLE II

COMPOSITION OF DRY AND WET CULTIVATED RICES

Percentages on fresh basis.

Mode of cultivation	Moisture	Proteins (crude)	Total minerals	P_2O_5	Ether extractives
	%	%	%	%	%
DIRTY MODAN					
Transplanted, swamp	12.0	10.77	1.470	0.713	2.33
Broadcast, swamp	12.5	9.45	1.412	0.640	2.41
Broadcast, dry	11.3	8.17	1.236	0.642	2.75
BLACK MODAN					
Transplanted, swamp	11.8	10.26	1.460	0.730	2.23
Broadcast, swamp	11.8	9.61	1.434	0.700	2.35
Broadcast, dry	11.3	8.35	1.266	0.639	2.60
KATTA MODAN					
Transplanted, swamp	12.4	10.00	1.474	0.695	2.23
Broadcast, swamp	11.4	9.06	1.314	0.690	2.32
Broadcast, dry	11.4	7.87	1.334	0.624	2.66

The results show that, in every case, irrigated transplanted rice was the richest in proteins and minerals, while dry rice generally contained the lowest percentages of these constituents, broadcast irrigiated rice being intermediate in composition between the two. A reverse relationship appears to hold in regard to the ether extractives of the different specimens.

Growth experiments: Feeding experiments with groups of young rats, four to five each, were carried out with the above rice samples with a view to finding out the rates of growth induced by them. The

technique of feeding was the same as that described in the previous publication (Sreenivasan, 1942). The animals, 30 to 35 days old, were given sufficient quantities of the test samples of rice (autoclaved) in the form of pan cakes, together with small uniform doses of cod-liver oil (0.05 g. daily) and marmite (2 cc. of 5% extract daily). Periodic growth charts were maintained for each animal in the different groups.

TABLE III

AVERAGE WEEKLY GROWTH RATES

Group	Mode of cultivation	Number of rats used	Average weekly increase in wt. in g. during		Remarks
			7 weeks	14 weeks	
			g.	g.	
		DIRTY MODAN			
1	Transplanted, swamp	4	8.14	6.50	—
2	Broadcast, swamp	5	6.86	5.43	—
3	Broadcast, dry	5	5.86	4.64	One rat died on 62nd day.
		BLACK MODAN			
4	Transplanted, swamp	4	7.43	6.07	One rat died on 18th day.
5	Broadcast, swamp	4	6.71	5.36	—
6	Broadcast, dry	4	5.43	4.36	—
		KATTA MODAN			
7	Transplanted, swamp	3	7.00	5.64	—
8	Broadcast, swamp	4	6.28	5.00	—
9	Broadcast, dry	4	4.71	3.79	One rat died on 76th day and another on 92nd day.
Standard error per sample:			0.30	0.20	
Significance groups:					
Without casein		1 4 7	2 5 8	3 6 9	
With casein		1 4 7	2 5 8	3 6 9	

After a period of seven weeks, when the rates of growth began to decline, the protein level of the food was slightly raised by adding 3% by weight of the diets as light white casein and the experiments continued for another seven weeks. It was assumed that such a supplement will not materially affect the differences in growth rates due to the rice samples. The average weekly growth rates are given in Table III and the growth charts in Figures 1 to 3.

The number of test animals used could not be increased, owing to practical difficulties. In a few cases, some of the animals had died during the experiment. Their weights were excluded from the calculations, as deaths were accidental. The results have been examined by Fisher's analysis of variance and the differences were found to be highly significant.

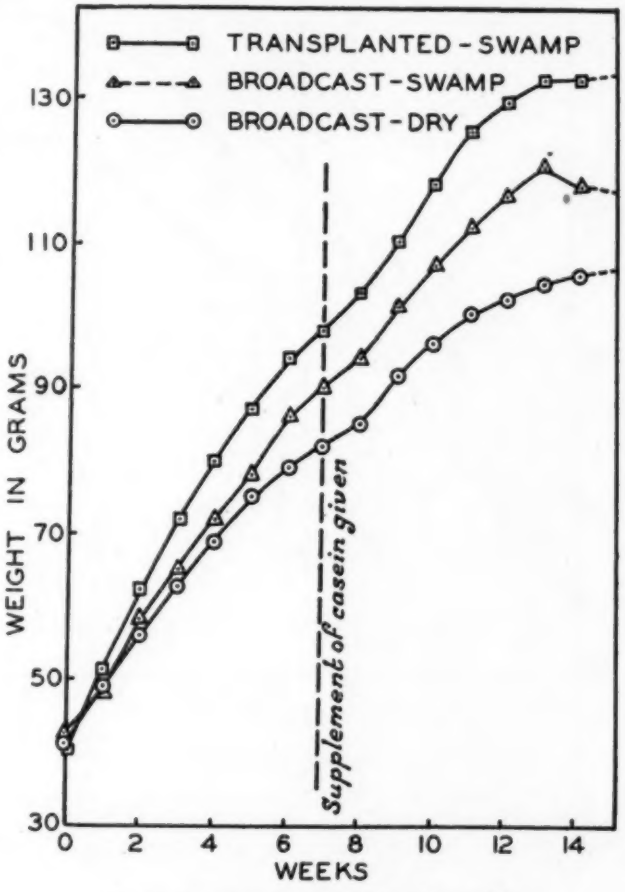

Fig. 1. Average growth rate, Dirty modan variety.

It may be seen that, for any particular variety, there is a significant difference in the growth-promoting qualities of the swamp and dry cultivated rices. The transplanted swamp rice had a definitely higher nutritive value than the transplanted broadcast rice, while the dry-grown rice had the lowest nutritive value. The differences were similar both before and after the casein supplements were given. These results are in accord with the chemical composition (Table II) and support the earlier

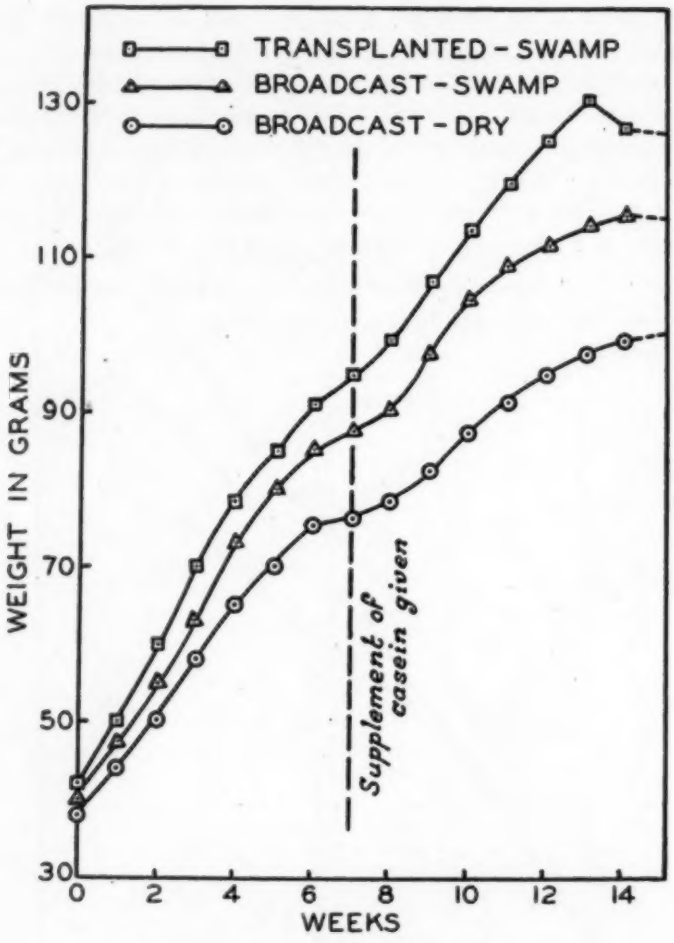

Fig. 2. Average growth rate, Black modan variety.

observation that growth is essentially related to the chemical composition, particularly the protein and mineral contents (Sreenivasan, 1942).

Discussion

Independent histological examination of the above samples have been carried out by the Paddy Specialist at Coimbatore, Madras, and it has been observed that there is a significant increase in the aleurone layer in favour of the swampy sample in all the cases (Ramiah and Mudaliar, 1939). In other work carried out by the senior author of this paper, it has been shown that when the rice plant is grown in swamp and in dry soil, the grain absorbs greater quantities of mineral nutrients in the

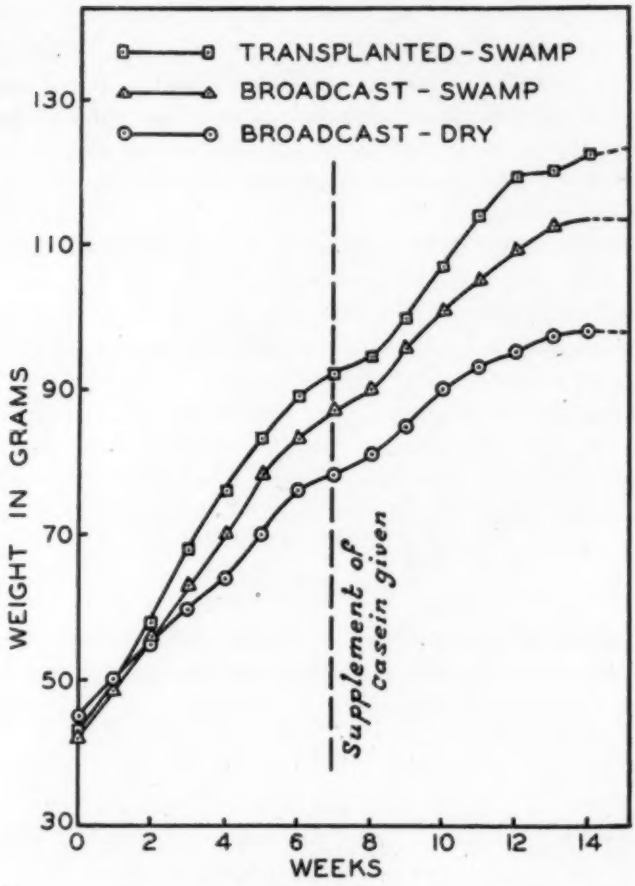

Fig. 3. Average growth rate, Katta modan variety.

former case than in the latter (Sreenivasan, 1936). Not only is the total intake of nutrients more in the swamp-grown rice but also the actual percentage of the mineral constituents in the grain.

Together with the fact that the yield of paddy is also increased nearly two to three times under the conditions of the swamp soil as compared to dry cultivation, these observations would mean that wet cultivation will not only yield better but also richer grains.

These results do not seem to support the findings of McCarrison (1928). It is possible that this technique of feeding was not such as to bring out the real differences between the nutritive values of the different rice samples. Thus, in his experiments, the test samples of rice formed only 12% of a basal diet which, according to the author, "provided a sufficiency of suitable protein, fats, inorganic elements and

water" and was besides "not devoid either of vitamin A or of vitamin B." In the present investigation, however, the diets, which were fed *ad libitum*, consisted essentially of the test samples of rice and yielded the pronounced differences observed between the different groups of animals. The results do not throw any light on the relative vitamin B_1 contents of dry and wet cultivated rices, as this factor was supplied from an external source.

The results of the wet transplanted and wet broadcast crops are perhaps the only ones that can be strictly compared because of differences in sowing dates and soil conditions with the dry crop. It is clear however that irrespective of the mode of cultivation, growth is mainly determined by the composition of the rice samples.

The practical application of the present inquiry is perhaps limited because dry cultivation of rice is more a matter of necessity than of choice and the farmer will gladly forsake it—if only for greater yield—as soon as he can get a supply of water necessary for wet cultivation.

Summary

The chemical composition and nutritive value of the protein and mineral constituents of three different rice varieties grown under (*a*) irrigated, transplanted, (*b*) irrigated, broadcast, and (*c*) dry, broadcast conditions have been studied.

Irrigated rice, irrespective of variety, was found to be richer in protein and mineral contents than dry cultivated rice; in the former case, transplanted rice contained higher percentages of these constituents than broadcast rice. A reverse relationship holds in regard to the fat contents of the different specimens.

In experiments on the growth of young albino rats fed with the different rice samples as the sole source of proteins and minerals, it was found that dry cultivated rice was least nutritious compared to wet cultivated, transplanted rice. Irrigated broadcast rice was intermediate in nutritive value between dry and irrigated, transplanted rices.

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THE RIBOFLAVIN CONTENT OF CEREAL GRAINS AND BREAD AND ITS DISTRIBUTION IN PRODUCTS OF WHEAT MILLING[1]

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(Read at the Annual Meeting, May 1941)[2]

Vitamin B_2 or riboflavin is one of the important members of the B-complex required for adequate human nutrition. Although cereal grains are not abundantly endowed with this food accessory, the consumption of liberal amounts can make an appreciable contribution to the dietary requirements. The magnitude of this contribution will depend to a large extent on the form in which the cereal is consumed. It is the purpose of this paper to present some of the data which have been obtained from the authors' analyses of a variety of cereals and cereal products.

In contemplating the vitamin B_2 analyses of such materials one is confronted with the problem of selecting an adequate method. Several procedures are available, including those which depend on the growth-promoting properties of this vitamin and those which measure its color or its fluorescence in ultraviolet light. Growth promotion can be measured by the use of rats as in the method of Bourquin and Sherman (1931) or one of its modifications, by the chick method of Norris *et al.* (1936), or by the microbiological method of Snell and Strong (1939). Since growth depends on a wide variety of factors,

[1] Paper No. 33, Journal Series, General Mills, Inc., Research Laboratories.

[2] Subsequently to the presentation of this paper at the Omaha meeting the microbiological method was modified to eliminate certain factors which adversely affect the assay results. The data presented herein were obtained by this modified procedure.

none of these methods is entirely specific and their successful operation requires careful adjustment of other growth-effecting substances which may be present in the samples being assayed.

Colorimetric and fluorometric methods offer certain advantages over growth methods in that they are somewhat more specific. This is especially true of fluorometric procedures such as that devised by Hodson and Norris (1939). An added advantage is the rapidity with which analyses can be made. For cereals, however, these advantages are in many instances lost due to the presence of interfering materials which prevent accurate measurement of the color or fluorescence of riboflavin. Conner and Straub (1941) have recently reported on a modification of Ferrebee's method (1940), which separates the interfering substances by adsorption of the flavin on "supersorb."

The Committee on Assay of Foods of the American Public Health Association (1941) has recently reported on the status of vitamin methods. They point out the absence of any officially recognized method for riboflavin and refer to the efforts of the Association of Official Agricultural Chemists to find satisfactory procedures. Kemmerer's (1940) report on this subject describes the results of preliminary collaborative studies.

Discussion of Microbiological Method

In the present study the authors have employed the microbiological method proposed by Snell and Strong (1939). Aqueous extracts of the cereals were added to the appropriate medium and inoculated with a culture of *Lactobacillus casei*. The amount of lactic acid produced after 48 hours was taken as the measure of the riboflavin content.

The practice of using extracts of cereals for the assay is in accordance with the originally proposed method. In view, however, of the possibility that extraction may fail to remove the riboflavin completely, a preliminary study was made in which the finely divided sample was added directly to the culture medium. Under these conditions appreciably higher values were obtained suggesting that the extraction was incomplete and that assays of extracts were too low. This seemed to be confirmed by the observation that the residues remaining after extraction exhibited a small but measurable riboflavin content.

In an attempt to make the extraction more quantitative, dilute hydrochloric acid was employed and preparations of diastase were added to break down the starch components. In most cases this technique failed to give higher values than those obtained by aqueous extraction. In fact, lower values were normally obtained.

This failure to increase the riboflavin content of extracts under more drastic conditions indicates that extraction may not be a major problem in the assay method. If this is true it then becomes desirable to find another explanation for the high results obtained by direct assay of the solid material.

In carrying out the microbiological method varying amounts of the cereal or extract are added to different tubes of the culture medium. These amounts are adjusted to give growth responses which fall within the range of linearity shown by varying quantities of pure riboflavin. The flavin content is then determined by reference to the quantity of pure riboflavin required to produce the same amount of lactic acid. Values obtained for the different levels should agree within 10%.

When the direct assay is applied to cereals a linear response to different quantities sometimes fails to be observed even though the growth range falls within that exhibited by the standard flavin solutions. We have referred to this as a "falling off" phenomenon since with increasing quantities the assay values become correspondingly lower. The magnitude of the difference between the highest and lowest levels is often greater than the desired 10%.

If it is concluded that the lower results are due to the presence of substances which inhibit the growth of the organisms the higher values would then appear to be more nearly correct. Such an assumption may, however, seriously affect the results obtained with cereal grains and leads to improbably high values in the case of products such as mixed feeds. This introduces a serious question as to the validity of assuming that the higher value is the correct one.

Recovery experiments with added riboflavin do not appear to provide the answer to this problem. While frequently more than 100% recovery is obtained, this does not necessarily indicate that growth-stimulating substances are present. When flavin is added to the sample a smaller quantity must be taken for the assay in order to keep the total flavin content within the linear range of the flavin standards. Where the "falling off" phenomenon is considerable, the smaller sample will give a higher value and account for the high recovery observed. It seems possible that some cereal products may contain nonflavin substances, some of which stimulate and others which inhibit the growth of the organisms under the assay conditions. The relative concentrations of these stimulators and inhibitors may decide their relative influences on lactic acid production.

When aqueous extracts are used instead of the solid sample the "falling off" phenomenon, though still quite evident, is of considerably less magnitude while in the case of aqueous extracts which have been treated with diastase this phenomenon almost entirely disappears.

For this reason there is a preference for the use of extracts rather than the solid samples, despite the fact that lower values are obtained.

Recent studies by the authors (1941) have shown that starch has a marked stimulatory effect on the growth of *Lactobacillus casei* and that its presence in cereal extracts results in fictitiously high riboflavin analyses. In white and whole wheat flours this effect can be removed by treating their aqueous extracts with takadiastase. Riboflavin values thus obtained are very similar to those determined by fluorometric methods. This agreement between two entirely different analytical procedures greatly increases the probability that the values reported herein are substantially correct.

All the data presented in this paper were obtained from extracts prepared by autoclaving the samples with water and digesting the resulting suspensions with takadiastase. Under these conditions the "falling off" phenomenon noted above almost entirely disappears and duplicate analyses consistently agree within 10% over the entire range of the different levels employed.

Riboflavin Content of Cereal Grains

During the course of the investigations a large number of samples of whole grains was examined. One series in particular, shown in Table I, was of considerable interest since it represented six different varieties of wheat, each of which was grown at four different locations.

TABLE I

RIBOFLAVIN CONTENT OF SPRING WHEATS

Variety	Location grown				Average
	St. Paul	Morris	Waseca	Crookston	
	μg per gram				μg per gram
Renown	1.15	1.25	1.15	1.15	1.17
Rival	1.05	1.05	1.10	1.05	1.06
Pilot	1.30	1.25	1.25	1.15	1.24
Thatcher	1.25	1.20	1.20	1.20	1.21
Ceres	1.20	1.20	1.25	1.15	1.20
Marquis	1.30	1.40	1.30	1.30	1.32
Average	1.21	1.23	1.21	1.17	1.20

While the number of samples involved is too small to justify definite conclusions it is believed that certain trends are indicated. It will be observed that the averages for the different locations are practically identical, suggesting that any environmental factors at each of the stations are sufficiently constant to be without effect.

On the other hand differences occur between the averages of some of the varieties and these differences are quite similar at each station. Thus Rival contains the lowest amount of riboflavin and Marquis the highest.

These observations are in marked contrast to those reported for thiamin on the same samples (Nordgren and Andrews, 1941). The thiamin content was found to be greatly influenced by environment, while differences between the varieties were not significant.

Table II gives the results obtained on other samples of wheats.

TABLE II

RIBOFLAVIN CONTENT OF WHEATS

Description	Riboflavin	
	Average	Range
	μg per gram	
Soft wheats:		
15 misc. varieties	1.17	1.05–1.30
Kawvale (3 samples)	1.17	1.00–1.30
Hard winter wheats:		
Turkey (7 samples)	1.22	1.15–1.30
Tenmarq (7 samples)	1.21	1.15–1.30
Chiefkan (6 samples)	1.09	1.00–1.20
Blackhull (7 samples)	1.17	1.10–1.25

The values obtained for these soft and hard winter wheats are very similar to those found in the hard spring wheats and suggest a greater constancy in riboflavin content than was found in the instance of thiamin. No effect of environment was observed and varietal differences are not particularly significant. Of the four hard winter varieties there is a trend toward lower values in Chiefkan but the magnitude of these differences is only about 10%.

A comparison of wheat with other grains can be obtained from the data given in Table III.

Barley, oats, corn, and rye are all quite similar to wheat in riboflavin content. The practically identical values obtained for the

TABLE III

RIBOFLAVIN CONTENT OF CEREAL GRAINS

Grain	Riboflavin	
	Average	Range
	μg per gram	
Yellow corn (13 varieties)	1.40	1.30–1.50
White corn (5 varieties)	1.38	1.30–1.50
Barley (6 varieties)	1.21	1.05–1.50
Oats (5 varieties)	1.30	1.10–1.45
Rye (6 varieties)	1.43	1.30–1.65

white and yellow corn are interesting since it is frequently assumed that the yellow varieties contain appreciably more vitamin B_2.

Riboflavin Content of Products of Wheat Milling

The products obtained from the milling of grains differ widely in their content of riboflavin, thus demonstrating the nonuniform distribution of this food accessory in the whole kernel. Table IV presents the values obtained from the analyses of the products separated in the milling of wheat.

TABLE IV

DISTRIBUTION OF RIBOFLAVIN IN PRODUCTS OF WHEAT MILLING

Mill product	Percent of wheat	Riboflavin	Percent of total riboflavin in wheat
	%	*μg per gram*	%
Patent flour	65.0	0.34	20.5
1st clear	5.5	0.62	3.2
2nd clear	4.5	1.85	7.7
Red dog	4.0	3.80	14.1
Shorts	12.5	2.80	32.5
Bran	8.5	2.80	22.0
Wheat	100.0	1.00	100.0

About two-thirds of the total riboflavin is contained in the feeds—red dog, bran, and shorts. The patent flour contains about one-third as much as the wheat from which it was milled. In many respects these relationships are very similar to those of vitamin B_1 except that patent flours contain a larger percentage of the total riboflavin in the wheat kernel.

No value is being reported for wheat germ since microbiological assays of this product are not yet regarded as satisfactory. The use of takadiastase in the preparation of extracts does not remove the "falling off" tendency and for this reason the assay values are probably in error.

The distribution of riboflavin in the various parts of the wheat berry is shown graphically in Figure 1. The riboflavin is represented by the solid line and for purposes of comparison the thiamin, shown by the dotted line, is also given. The similarity of the two curves suggests that the same mechanism is responsible for the way in which they are distributed throughout the wheat kernel.

Riboflavin Content of Bread

Because a large fraction of the flour milled from wheat is consumed in the form of bread it is important to examine this product, especially since some of the ingredients other than flour are important sources of

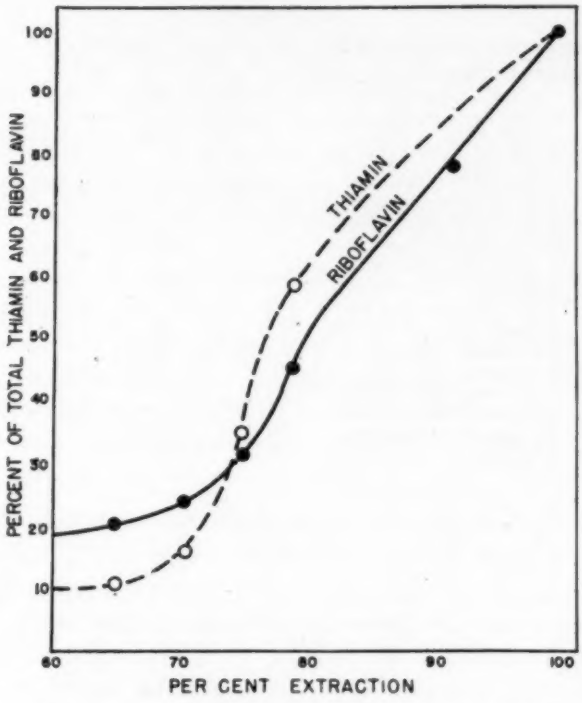

FIG. 1. Distribution of thiamin and riboflavin content in wheat.

riboflavin. Three series of breads were prepared using 2% of yeast and, in addition, salt, sugar, shortening, and yeast food. The first series was made from patent flour, the second from the same flour containing 2 μg per gram of added riboflavin, and the third from whole-wheat flour. In each series increments of nonfat milk solids ranging from 0% to 9% were added. Table V gives the results of riboflavin assays of these products.

In many instances the agreement between the calculated and found values is extraordinarily good. Such agreement must be regarded as entirely fortuitous, since the method is not so accurate as these values might indicate.

It is apparent that there is no measurable loss of riboflavin in the production of bread. This is to be expected since flavin is quite stable to heat. On the other hand, serious losses will occur if the bread is unduly exposed to light. Early in the investigations it was found that samples of sliced bread which were allowed to dry on benches in the laboratory contained much less than the expected amount of riboflavin. Subsequent studies showed that these losses can be as high as 50% even in the absence of direct sunlight. On the other hand,

TABLE V

THE RIBOFLAVIN CONTENT OF BREADS

Bread	Calculated	Found
	μg per gram fresh bread	
White:		
No nonfat milk solids	0.60	0.53
3% nonfat milk solids	0.99	0.92
6% nonfat milk solids	1.37	1.31
9% nonfat milk solids	1.75	1.74
White (1.27 μg of added flavin):		
No nonfat milk solids	1.84	1.78
3% nonfat milk solids	2.22	2.08
6% nonfat milk solids	2.60	2.53
9% nonfat milk solids	2.98	2.77
Whole wheat:		
No nonfat milk solids	1.02	1.16
3% nonfat milk solids	1.40	1.41
6% nonfat milk solids	1.79	1.80
9% nonfat milk solids	2.17	2.09

losses in whole wheat bread, under the same conditions of drying, are considerably less. In one experiment where a 40% loss occurred in white bread only a 10% loss was found in the whole wheat variety. It is very important in preparing bread samples for assay to carry out the drying operation in the dark.

Table VI gives the riboflavin content of the ingredients which contributed to the total in the above breads.

TABLE VI

THE RIBOFLAVIN CONTENT OF BREAD INGREDIENTS

Ingredient	Riboflavin
	μg per gram
Patent flour	0.35
Whole wheat flour	1.0
Yeast	31.1
Nonfat milk solids	20.6

In the case of the bread made from patent flour, the flour, 2% of yeast, and 3% of dried milk contributed appreciable amounts of riboflavin. As the amount of milk was increased its contribution became correspondingly greater. In practice the contributions of the ingredients will vary, since differences in riboflavin content occur. The yeast used in this study was somewhat higher than most fresh yeasts which have been examined, since usually a value of about 20 μg per gram has been found. The nonfat milk solids also vary. The analyses of a large number of samples have given values ranging from a low of about 16 to a high of 25. The average was about 19 μg per gram.

Experimental

The microbiological method employed for the assay of riboflavin is essentially that described by Snell and Strong (1939). Preparation of the extracts for the assays was carried out by autoclaving 5- to 10-gram samples of the finely ground cereal with 90 ml of distilled water at 15 lbs pressure for 15 minutes. After cooling to about 50°C, 5 ml of 6% takadiastase solution was stirred in and the suspension allowed to stand for 1 hour. It was then made up to 100 ml with distilled water and clarified by centrifuging. All operations were carried out in a darkened room.

The takadiastase employed in preparing the extracts contained riboflavin. The amounts were, however, too small to significantly affect the analytical values. Preparations of takadiastase should be examined to insure that serious errors are not introduced from this source.

Summary

The riboflavin content of a variety of cereals and cereal products has been determined by the microbiological assay method. There is a close parallelism between the thiamin and riboflavin contents of wheat and its products of milling. No measurable loss of riboflavin occurs in the baking of bread but large losses are found when slices of bread are unduly exposed to light.

Acknowledgments

The authors are indebted to Mr. B. A. McClellan for preparing the samples of bread. The samples of cereal grains were kindly supplied by Dr. C. H. Bailey, University of Minnesota; Dr. John H. Parker, Kansas Wheat Improvement Association; Dr. B. H. Thomas, Iowa State College; and Dr. C. A. Lamb, Ohio Agricultural Station.

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ON THE MULTIPLE AMYLOSE CONCEPT OF STARCH. II. AMYLOPECTIN AND AMYLOSE[1]

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In a preceding paper (Kerr and Trubell, 1941) several of the more modern concepts favoring the view that starch is composed of only one type of molecule have been reviewed and shown to be untenable for corn starch. Therein it was demonstrated that there are at least two fractions, separate and distinct from each other, the one called simply amylose for the present, and the other gamma-amylose. In this paper it is proposed to show that the behavior of the starches is not explainable as consisting only of varying ratios of amylose and gamma-amylose, but that the starches are fabricated from more than two amyloses.

The bulk of the starch granule has been at various times during the last half century (and even up to the current year) conceived as divisible into two main constituents: amylose and amylopectin, or alpha- and beta-amylose, or fractions otherwise designated. The first question to be discussed is whether gamma-amylose is identical with the fraction frequently called amylopectin. A second issue is to consider the existence of amylopectin, itself, the presence of which as a chemical entity has recently been seriously questioned.

To introduce the discussion which follows, it is our desire to point out first that much of the confusion which has followed from apparently contradictory evidence in this field of work results largely from poor definition and from the promiscuous use of terms without due reference to the original concept for which these terms had definite meanings. For example, Taylor and Schoch (1933) deny the existence of amylopectin in potato starch, inasmuch as they could find no fraction of potato starch which corresponded to corn alpha-amylose, their equiva-

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lent of amylopectin. Moreover, they were unable to demonstrate that the fractionation of potato starch, by use of a cataphoresis cell, led to a separation of combined phosphorus in the component which migrates to the positive electrode. Samec (1927) had claimed such a separation and has characterized amylopectin as a phosphoric acid ester. From their work Taylor and Schoch conclude that potato starch is all soluble amylose—that is, contains no amylopectin. The errors involved in this logic are worth considering, inasmuch as many of the arguments denying the existence of hypothetical amylopectin in all starches follow an analogous pattern.

Taylor revived the terms "alpha"- and "beta"-amylose proposed by A. Meyer, stating that the differentiation rested primarily on solubility. Later Taylor and Iddles (1926) added to this concept their observation that the fatty acids of corn starch are concentrated in the alpha-amylose fraction. In the meantime Samec had developed his amylophosphoric acid theory for amylopectin. These investigators showed independently, moreover, that their hypothetical carbohydrate esters, alpha-amylose and amylopectin, migrated in a cataphoresis cell. From the evidence, Taylor must have considered alpha-amylose the equivalent of amylopectin, for Taylor and Schoch (1933) use the two terms synonymously.

In the first place Meyer himself was not satisfied with the concept of alpha- and beta-amylose, certainly not in using solubility as a prime basis of distinction, for in a historical review of the latter's work by Samec (1927) it is made evident that Meyer's prime basis of differentiation for alpha-amylose became finally its greater resistance both to diastatic enzymes and to acid.

The introduction of the concept of retrogradation by Maquenne at about this time was most opportune, for it gave a plausible explanation to the inconsistent results of Meyer, because of which results Meyer had become convinced that there was no fundamental difference between his two fractions. It was now apparent that most of Meyer's resistant amylose fractions contained retrograded amylose in varying amounts.

Unfortunately, however, Maquenne and Roux (1905) appear to have drawn the sweeping conclusion that all insoluble and resistant fractions of starch (amylocellulose, alpha-amylose, etc.) are but physical modifications of one substance, which was designated later simply as amylose. Nevertheless they were unwilling to concede that starch is one substance, chemically. To account for the fact that a part of starch is converted only as far as dextrin by diastase, whereas their preparations of amylose were believed to convert completely to maltose, the term amylopectin was introduced by these investigators (Maquenne

and Roux, 1905) to name the nonsaccharifiable portion. The choice of name was influenced by the fact that their saccharifiable fractions of starch did not gel, from which it was assumed that the fraction which formed dextrin was probably the same component which imparts to starch the property of paste formation. It should be clear, therefore, that this hypothetical amylopectin is not the equivalent of alpha-amylose. Indeed, the former has no counterpart in the alpha-beta-amylose nomenclature.

Not only Taylor, but many of his contemporaries who were ardent supporters of the multiple-amylose concept have inadvertently done as much as their opponents to cast suspicion on this concept, in the confusion which has resulted from the manner in which they have used these terms, with little consideration for the original concept, in designating their fractions.

Other workers, as for example Hanes (1937), appreciate fully the original distinctions drawn between amylose and amylopectin, but conclude that a differentiation on this basis is faulty, since it rests on a misconception of the mechanism of starch enzymolysis. Possibly it does. More will be said in this connection in our discussion which follows. Possibly, if starch is a nonhomogeneous mixture, some other basis for differentiating these fractions will be found to be the true one. But if we are to emerge from the confusion into which this field of work has drifted, it should be obvious that the revival of outmoded terms or the inaccurate use of other terms to describe various fractions of starch should first be discontinued.

Amylopectin as a Constituent of Starch

Tychowski (1937), accepting the modified procedures of Ling (1928) for estimating amylose, wherein barley diastase is used in place of malt diastase (which contains a factor strictly dextrinogenic and is incapable of producing maltose—Hanes, 1937), has more accurately determined the percentages of maltose formed from various starches as follows: potato, 63%; wheat, 65%; rye, 63%; barley, 62%; corn, 59%; and rice, 59%. From this and a further study (1937) he estimates that the amylose content of all starches is only approximately 58% of the total starch. The remainder is claimed to be amylopectin.

We have attempted first to determine more accurately this amylopectin value, while at the same time answering a fundamental objection to this method of analysis, and finally, to point out that gamma-amylose is not this hypothetical constituent. It has long been supposed that retrograded amylose is resistant to the diastases and it is not inconceivable that a part of the amylose as it exists in the starch granule is already in the retrograded form, in the sense that in ordinary

gelatinization procedures it is not sufficiently hydrated, dispersed, or otherwise conditioned to render it readily convertible. Thus, the failure of starch to be readily converted entirely to maltose might be explainable on a basis of preretrogradation of a part of the granule.

First of all, therefore, samples of corn, potato, and tapioca starches were thoroughly dispersed in 0.67*N* KOH, neutralized, and immediately converted with barley diastase in accordance with procedures given in the "Experimental" section. The results of this conversion are given in Table I.

TABLE I

DIASTATIC CONVERSION OF ALKALI GELATINIZED STARCHES

Conversion time	Percent of dry substance starch converted to maltose		
	Corn	Tapioca	Potato
min	%	%	%
5	14.87	17.8	18.15
15	33.2	36.6	35.6
45	46.6	47.2	46.7
75	51.1	51.9	50.5
105	53.8	55.7	53.8
180	56.6	59.2	58.6
960	60.0	64.4	66.5

These values, when plotted graphically, show that with complete dispersion there still exists a definite change in the course of the reaction between 55% and 60% conversion. Up to this point the reaction curves for the three starches are very nearly superimposable, but beyond this point there is a secondary reaction which cannot be entirely explained, as has been attempted, by ascribing the continued rise in reducing value to traces of alpha-amylase in the enzyme preparation used—acting on a common substrate in each case, the dextrins from amylopectin. The course of this latter reaction is also influenced by the composition of the substrate, whether it is principally amylopectin dextrins or whether, as we intend to show, it is the latter plus gamma-amylose.

While our results in general agree with the experimental values of Tychowski, it is felt that a refinement in analyzing and interpreting these results would lead to a more accurate estimation of the actual amylose and amylopectin contents of these various starches by this general method.

First of all, in Figure 1 the logarithm of the percent starch converted into maltose is given against conversion time which shows strikingly the change from initial reaction rate to final. If these two reaction rates were extrapolated to their point of intersection, we might more

precisely locate the point at which the first reaction ceases and the second begins.

However, accepting the multiple-amylose concept, to be consistent one should presuppose that the reduction values shown in the first part of the curve are the sum of those from maltose, which originate from amylose through no intermediate stages, plus reducing values from higher products resulting from one or more independent reactions—the simultaneous degradation of amylopectin, gamma-amylose, etc.

With these assumptions the latter stage of the reaction should be in progress even during the initial reaction and the reducing values of

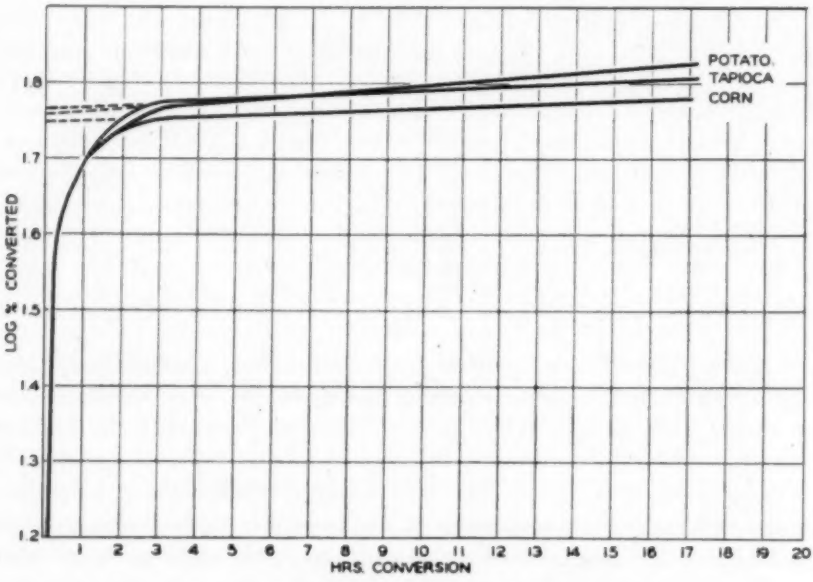

Fig. 1. Curves for potato, tapioca, and corn starch.

the products formed should be subtracted from the total found at completion of the first reaction in order to estimate the amount of amylose, only, converted. This deduction can be accomplished by extrapolating the logarithmic curve for the second reaction to zero time and noting the amount of maltose corresponding to this intersection point.

Figure 1 shows the logarithmic curves for corn, tapioca, and potato starch, from which we estimate that the amylose content of these starches are 55.8% for corn, 57.6% for potato, and 57.8% for tapioca.

We have previously shown (Kerr and Trubell, 1941) that tapioca and potato starches contain negligible quantities of gamma-amylose, less than 1% for tapioca and an amount for potato too small to isolate,

and we have estimated that corn starch contains approximately 10%. It should be evident, therefore, that the fraction, gamma-amylose, cannot account for all of that portion of starch which does not convert to maltose. There is a portion which in contrast to gamma-amylose is speedily converted to a more or less soluble, dextrinous material. This latter fraction, by definition amylopectin, should comprise then about 34% of the weight of corn starch, 41% of tapioca, and 42% of potato.

To show that dispersion in alkali is sufficient to condition retrograded amylose for conversion, the following experiment was attempted:

Tapioca starch was alkali-gelatinized as had been done previously and now neutralized to pH 4.5. It was allowed to retrograde under toluol for 10 days at 4°C. The entire mixture was then treated with barley diastase for 24 hours, in accordance with our procedures previously given (Kerr and Trubell, 1941) for preparing gamma-amylose. The insolubles were centrifuged and washed free of reducing substances in the centrifuge, and these resistant insolubles were now dissolved in 0.67*N* KOH at 25°C. After 30 minutes HCl was added to adjust the pH to 5.0 and after one hour's standing the gamma-amylose precipitating was centrifuged out. The centrifugate was immediately converted with barley diastase at pH 6.0 and 47°C; the maltose formed from time to time was determined by alkaline ferricyanide reduction and the percent of dry solids present converted to maltose was calculated (Table II).

TABLE II

CONVERSION OF RETROGRADED TAPIOCA STARCH

Conversion time	Dry substance converted to maltose
	%
15	41.5
30	59.4
75	70.8
150	72.9
210	73.1

It is apparent that while the conversion limit for this material is definitely higher than for whole starch, it is still incomplete, for when this conversion was plotted as was done for parent starch, a conversion limit of 71.6% was estimated. This latter result may be partly explainable on the basis that all of the more resistant gamma-amylose failed to precipitate from the solubilized retrograded amylose in one hour. However, whatever the explanation, it is apparent that retrograded amylose will convert with diastase after redispersion in

alkali and will do so at an accelerated rate, as compared with whole starch. It does not seem likely, therefore, that we can entirely explain the abrupt change in the course of the diastatic curve for conversion on a physical or physical-chemical basis, alone.

To settle the above question more conclusively, however, requires the clarification of many uncertainties. To obtain pure retrograded amylose presupposes that we obtain a pure amylose, procedures for which are still lacking. If we assume that amylose may be purified by the process of retrogradation, we limit ourselves to the proposition that only amylose retrogrades, in which case a better definition of retrogradation than the varied ones now prevalent is required.

A definition of retrogradation as the change of a soluble, non-resistant fraction of starch into an insoluble, more resistant form would include the precipitation from solution of corn gamma-amylose either by lowering the temperature or changing the pH of the solution. To exclude the above phenomena by limiting retrogradation to an irreversible change is not in accordance with the experiment above given. And to limit retrogradation to progressive precipitation from comparatively dilute solutions at lower temperature and normal acidities would ignore our experiments on gamma-amylose, in which it is seen that a distinction on this basis is only relative.

Amylose as a Constituent of Starch

Older methods of starch fractionation: The major problem before us, then, is the separation of amylose—that is, the separation of a fraction that will convert straightforwardly to maltose with barley diastase. In addition to the partially successful one, just given, by retrogradation, we have tried several of the more commonly used and quoted procedures purporting to give such a separation, but with unsatisfactory results. These are electrophoresis of a dispersed starch paste, simple hot-water extraction of starch granules, and freezing and thawing of starch pastes. In addition, we have evidence that a fifth method (Samec, 1929), precipitation with alkaline earths, would be unsatisfactory. Thus we have found that gamma-amylose also forms a difficultly soluble barium salt which is not readily fractioned by progressive solubilization from the mixture precipitated. This experiment will be treated in greater detail in a following communication.

Amylose by electrophoresis was prepared by procedures used by Taylor and his coworkers, but using autoclaved corn-starch pastes in accordance with procedures elaborated by T. J. Schoch. Hot-water-soluble amylose was prepared, as recently suggested by Meyer, Bretano, and Bernfeld (1940). However, our yield was only about one-third of the 16.0% obtained by Meyer in one hour's extraction at

80°C. Three independent extractions gave us yields of 5.4, 4.9, and 6.0% respectively.

Amylose by freezing and thawing was prepared by the well-known procedures used by Ling and by Pringsheim and Wolfssohn. A 5% paste of tapioca starch was frozen for 16 hours at −10°C. The mass was then washed with water at 60°C. Most of the slimy insoluble residue was thrown out by centrifuging and the centrifugate filtered. The soluble amylose yield was approximately 61% for tapioca starch. Each product was converted by procedures given for obtaining conversion data in Table I.

TABLE III

CONVERSION OF AMYLOSE FRACTIONS

Conversion time	Dry substance converted to maltose		
	Amylose by: Electrophoresis	Hot water	Freezing and thawing
min	%	%	%
5	28.6	19.3	18.8
15	50.5	41.1	37.3
45	62.0	69.6	53.7
75	64.5	77.6	58.7
105	65.5	79.0	60.5
180	67.5	80.5	61.5
1200	71.0	85.0	66.9
Conversion limit	66.0	79.0	60.2

It is apparent from the above data, shown graphically in Figure 2, that only amylose by hot-water extraction has a significantly higher conversion limit than the parent starch. Amylose by cataphoresis has a measurably higher limit, but inasmuch as the paste for separation was made by autoclaving, this increase is perhaps understandable in view of the work cited by Hanes (1937, p. 198–200). The small yield of the amylose by hot-water extraction detracts from the significance of our results obtained and leaves these results open to the argument that we had extracted but a small quantity of amyloid material present in the granule in a state of incomplete synthesis to starch or material degraded from starch in the process of starch manufacture.

We do not agree with the last given interpretation for several reasons, but concede, however, that existing data do not support the view that approximately 60% of the weight of starch converted to maltose can be ascribed to the complete conversion of one distinct amylose. Conversely, then, it might appear that a more suitable interpretation would be that of Hanes, previously referred to, that the dextrins of beta-amylase conversion of starch are not the products from

a distinct component, amylopectin, but are, rather, the short-chain residues of a single and originally longer starch molecule. However, in examining this latter theory, the reason why the enzyme has greater difficulty in hydrolyzing the molecule as the chain becomes shorter is not satisfactorily explained. Moreover, since the concept of side chains or other enzymatic blocks has been subsequently introduced, the conclusion that these residues are necessarily of much lesser molecular magnitude has become even less convincing.

Great weight is attached by Hanes to end-group analysis by methylation, as indicative that these dextrins are 40% of the length of the

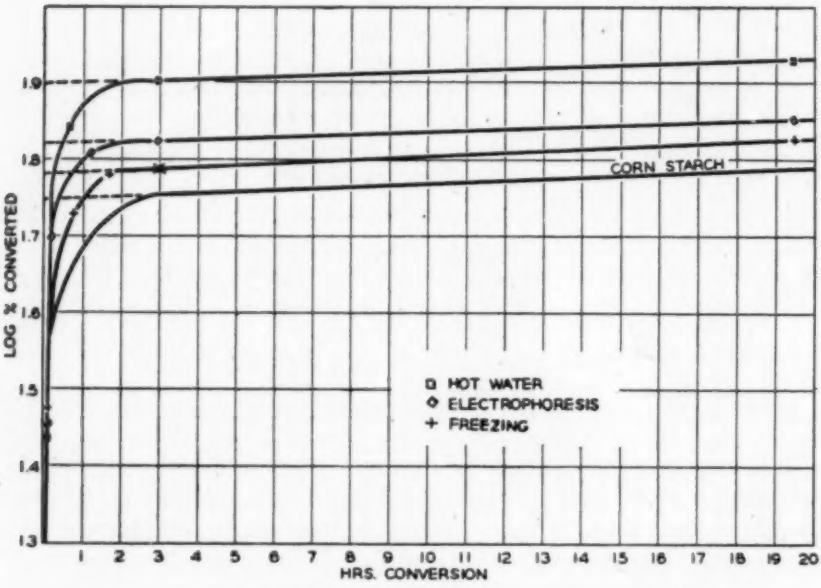

Fig. 2. Curves for amyloses by electrophoresis, hot water, and freezing.

starch molecule. However, even in arriving at an estimate of the original chain length of starch, Staudinger (1937) has pointed out that these results of end-group analysis may be interpreted differently. It is evident that it is possible to obtain the same distribution between tri- and tetra-methyl dextrose from a molecule many times larger than that given by Haworth, provided branching of the chain exists. And if starch possesses only one molecular configuration, as suggested by Hanes, it would be very difficult, except by supposing a side-branched structure, to account for the results of Freudenberg on the di-methyl products obtained from fully methylated starch (Freudenberg, 1940).

Moreover, the support given by Hanes to Haworth's concept appears to be less convincing, since Myrbäck (1938) has indicated by

other methods that these limit dextrins may approach the order of starch itself in molecular magnitude. While the absolute values of Myrbäck's results might be questioned, it appears probable that the values are relative.

With a reasonable doubt existing, therefore, as to the conclusions given by Hanes concerning Maquenne's hypothesis we attempted to fraction starch by two methods, given below, in order to shed further light on this problem.

Fractionation of starch by alcohol precipitation: Schoch (1941) has indicated that starch may be fractioned into dissimilar components by adding higher alcohols to autoclaved starch pastes and supercentrifuging the mixture.

To avoid the solubilizing effect of high temperatures on the gamma-amylose constituent and to avoid the criticism that prolonged autoclaving increases the convertibility of starch fractions, as claimed by Hanes, we used a homogenizer to effect dispergation of the starch; and to obtain greater ease in the precipitation of the pastes, we combined the use of butyl alcohol with the more soluble methyl alcohol. Our procedures, as detailed in the experimental section, give us two main fractions: one which is least soluble in alcohol-water mixtures and should contain nearly all of the gamma-amylose content of the starch plus noncarbohydrate residues and plus other amyloid material, and a fraction which is more soluble.

The last-named fraction is very interesting in that its alkali-labile number, determined according to Schoch and Jensen's (1940) modification of Taylor's procedures, is of a very low order, as low as the value for whole tapioca and potato starches, which themselves are only about one-half as alkali-labile as corn starch. Hence, to point out more clearly the possible chemical difference for constituents of this fraction, we determined and compared alkali numbers for the two fractions above mentioned, together with the alkali numbers for gamma-amylose, corn, tapioca, and potato starches. These values are given in Table IV.

TABLE IV

ALKALI NUMBER AND CONVERSION LIMIT OF STARCHES AND FRACTIONS

Starch, or fraction	Alkali No.	Yield	Conversion limit
		%	%
Alcohol, more soluble	5.7	55.6	56.6
Alcohol, less soluble	20.0	44.4	65.3
Corn starch (dioxane extracted)	11.7	—	59.4
Gamma-amylose	42.0	(11.55)	48.1
Alcohol, less soluble, less actual gamma-amylose content	12.7	32.8	71.0 (estimated)
Tapioca starch	4.7	—	—
Potato starch	7.8	—	—

Having demonstrated by alkali numbers that corn starch can be fractioned by alcohol precipitation, it would appear, from the following three considerations that there are at least three fractions in corn starch.

First, it was found that corn starch divided itself into 44.4% as less alcohol soluble and 55.6% as more alcohol soluble, and that the former fraction contained 26.1% gamma-amylose by our method of separation (Kerr and Trubell, 1941) or 11.55% of the weight of starch. This latter value agrees fairly well with our previous estimate that corn starch contains 10% gamma-amylose. Thus there is left 32.8% of the weight of starch, less soluble in alcohol, unaccounted for.

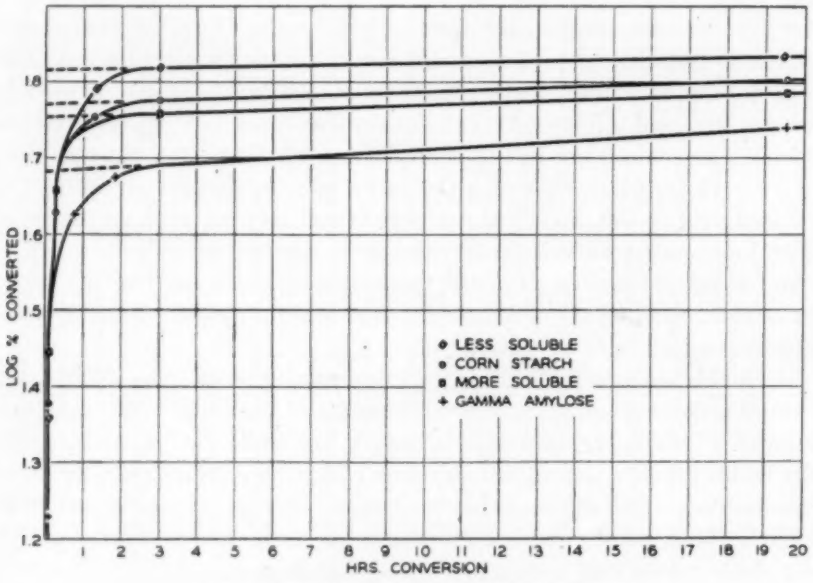

Fig. 3. Curves for conversions of various fractions.

Secondly, it is apparent that the alkali lability of corn starch cannot be accounted for as consisting of only gamma-amylose and the soluble fraction. For the alkali-labile number of the latter fraction is at least as low as 5.7, possibly lower if purified further, and assuming a composition of 1 part gamma-amylose to 9 of the soluble fraction, we arrive at a total value of only 9.3. It should be apparent then that there is room for a third fraction with an alkali number slightly higher than starch itself.

Lastly, to account for the alkali number of the less soluble alcohol fraction on the basis that it is only a mixture of one part gamma-amylose to three parts of the more soluble fraction involves an even wider discrepancy between found and estimated values, and points

more conclusively to the existence of a third component. Further differences in these fractions will now be pointed out. It will also be noted in passing, however, that the values obtained for the potato and tapioca starches leave little room for a component with as high an alkali number as gamma-amylose and we believe that this experiment explains, incidentally, the higher value for corn starch as compared to the two noncereal starches.

Diastatic analysis of starch fractions: The above-described fractions were converted with barley diastase in the manner already given and the conversions plotted in Figure 3, from which the limits of conversion given in Table IV were estimated. It will be observed that:

1. The soluble fraction converts to a slightly lower limit than whole starch. This material, as a whole, then, is not the hypothetical amylose of Maquenne.

2. The initial conversion rate extends further with the less soluble fraction than with starch or the other two fractions of starch.

3. Gamma-amylose, solubilized by alkali before conversion, reaches a conversion limit much sooner than the other fractions.

Knowing that the less soluble fraction is one-fourth gamma-amylose, it is apparent then that the remaining part of this fraction has a conversion limit of over 70%. It is possible, therefore, that were this portion freed of gamma-amylose and of impurities, probably of the more soluble type, a relatively high conversion limit would be found for this fraction, even of the order of that shown for Kurt Meyer's hot-water-extracted amylose.

It is significant, we believe, that our most readily converted fraction should be found in that part that is least soluble, whereas Meyer's amylose is apparently very soluble. It is our opinion that solubility is, therefore, a poor general criterion for amylose and the indications are that amylose may be a series of products related chemically but with varied physical properties. It is understandable then how one might be led to conclude as did Ling, that "amylose" would never be completely and quantitatively separated because only a part of it, as he expressed it, is in the "free" condition.

Separations by fractional precipitation of starch acetate: Although it is apparent that dissimilar fractions are obtainable from corn starch by fractionally precipitating a paste with alcohols, as judged by the diastase convertibility and the alkali labile number of these fractions, purification of these fractions appears to be difficult because precipitation is not clean cut. The precipitates are thrown out with great difficulty so that even after reprecipitation of these fractions one is not sure that considerable adsorption of the more soluble fraction on the less soluble has been avoided, and it is almost certain that a small

portion of the less soluble fraction remains colloidally suspended in the more soluble fractions.

To overcome these difficulties the fractional precipitation of starch triacetate was attempted by gradual additions of petroleum ether to solutions in chloroform in more or less the same manner that the acetates of the limit dextrins have been fractioned in our laboratory.

Both the preferred method for acetylation of corn starch, developed for this particular work, and the method of fractionation of the acetates are given in the experimental section.

Three distinct fractions were prepared. One was a fraction which swelled in chloroform to a gel-like nature and did not truly dissolve. This would correspond in superficial properties, at least, to an acetate fraction described by Hanes (1937, p. 124), purported to be an amylopectin acetate. A second fraction was obtained, soluble in chloroform

TABLE V

Fractions of Corn Starch Triacetates

	Yield	Acetyl	Beta-amylase conversion limit
	%	%	%
Least soluble fraction	34.4	41.9	57.5
Intermediately soluble fraction	52.25	43.0	75.2
Most soluble fraction	13.25	45.7	69.9
Parent starch triacetate	—	43.5	63.1

with a rather high viscosity therein but insoluble in mixtures of chloroform containing higher than 57.2% by volume of petroleum ether. It precipitated as a flocculent mass. A third fraction was obtained, soluble in chloroform-petroleum ether with a concentration of the latter higher than 57.2%. It precipitated over a broad range, but very nearly completely, however, at about 70% petroleum ether concentration. It deposited first as a gummy material which changed over to a refractive, granular powder when treated with petroleum ether, whereas treatment of the other two products with petroleum ether resembled the dehydration of a concentrated water solution of a dextrin with alcohol. Freed of their residual chloroform by washing with petroleum ether, they were dried with the following yields, as shown in Table V.

Acetyl groups were determined by the method of Murray, Staud, and Gray (1931) using pyridine as a dispersing agent, but this procedure did not provide a completely homogeneous reaction mixture, either for the starch triacetate or for the chloroform insoluble fraction. This is one factor, possibly, that accounts for the analyses showing values

less than the theoretical 44.8% acetyl. Another factor may have been the use of the whole starch triacetate, which contains approximately 1% noncarbohydrate impurities such as adsorbed or combined ash, protein, and lipid material not readily extractable by common organic solvents. These impurities might accumulate in the least soluble fraction and proportionally decrease the apparent acetyl value for this fraction.

Larger quantities of these fractions, together with the parent starch triacetate, were all deacetylated in the same manner, and after neutralization of the deacetylated reaction mixtures the regenerated

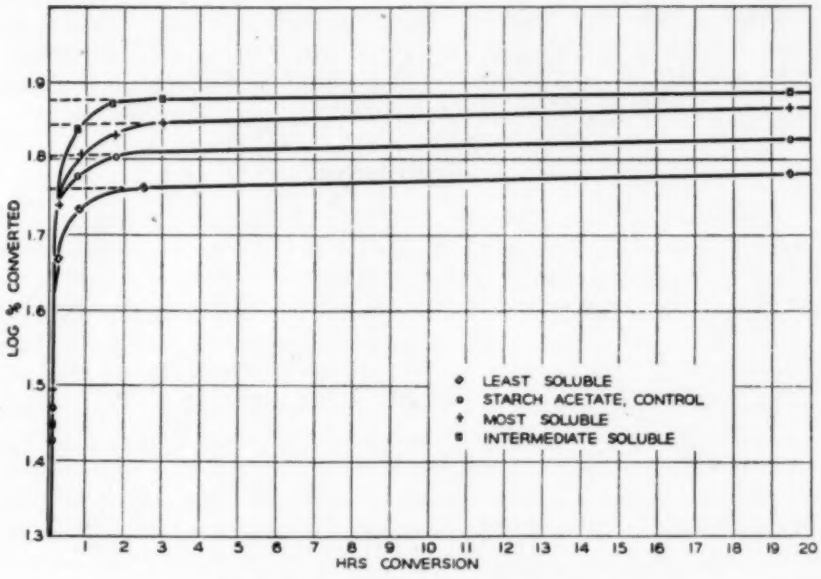

Fig. 4. Curves for conversions of various fractions.

carbohydrate fractions were precipitated by adding enough ethyl alcohol to result in a 50% (by volume) alcohol solution. After centrifuging, the precipitates were washed free of pyridine with alcohol, then with ether, and finally dried *in vacuo*. These products were completely dispersed in 0.67*N* NaOH, neutralized to pH 5.0 with HCl and converted with barley diastase by procedures identical to those used on the starches and other starch fractions (see experimental section).

The results of these diastatic conversions are given in Figure 4, from which the conversion limits shown in Table V have been estimated. It is apparent that even greater differences in convertibility result with these fractions than with starch paste fractioned by alcohol precipitation. It is to be noted that the fraction intermediately soluble in

respect to the acetate derivative, is now by far the most readily converted.

It is our belief that refractionation of this portion will yield components showing an even higher limit of conversion. Indeed, it is to be expected that each of the two more soluble fractions may in turn be further subdivided into their constituents by an extension or elaboration of the fractionation given.

With the theory of starch composition apparently becoming more complex, it would seem, therefore, that the major problem before us is the isolation in pure form of these several components, for which a new terminology seems to be very much needed. While Maquenne's amylose-amylopectin theory, which superseded the alpha-beta-amylose concept, has not as yet been shown to be an impossible one, it must be admitted that the theory is still highly speculative and possibly an oversimplification of the composition of the starches.

Experimental

Conversion of starch products with barley diastase: Two grams of product (dry basis) were wet with 10 ml of water to which was then added, with stirring, 20 ml of normal NaOH. Stirring was continued for 30 minutes at 25°C. One hundred ml of water was then added and the solution was carefully adjusted to pH 5.8 with HCl, using the glass electrode. The volume was then brought immediately to 250 ml, readjusting the pH, if necessary, and 2 ml of barley diastase (the extract equivalent to 0.5 g of barley) added as soon as possible after bringing the starch product solution to 47°C.

Aliquot portions were removed at definite intervals, pipetting directly into a mixture of sodium carbonate and ferricyanide and the maltose estimated according to the method of Gore and Steele (1935). The percentage of starch converted to maltose was then calculated after deduction of the value for a blank made by pipetting proportionate amounts of both enzyme and starch solution into the alkaline ferricyanide. The preparation of our barley diastase has been previously described (Kerr and Trubell, 1941).

Separation of starch components with alcohols: A 3% suspension of dioxane-extracted corn starch, at pH 5.5, was brought up slowly to 90°C, held for 30 minutes, and then cooled quickly to 65°C, whereupon it was immediately run through a two-stage homogenizer (Viscolizer, Junior) at 4,300 lbs of pressure. Under these conditions, dispergation of the starch appeared to be complete. The liquors emerging at 55°C were immediately saturated with normal butanol and allowed to stand overnight. Methyl alcohol was now added slowly with vigorous agitation until the content of the latter was 15% by volume and stirring

was continued 3 to 4 hours. The insolubles resulting were then centrifuged out and washed once in the centrifuge with a mixture containing approximately 75% water, 10% butanol, and 15% methanol.

The insolubles were further purified by resuspension in about 85% of the water used to make up the original starch paste and brought up to a boil slowly with very thorough stirring. As the paste cooled it was again saturated with butyl alcohol and after an hour or so at room temperature, methyl alcohol was again added to a content of 15% by volume, and allowed to stand overnight. Thereupon the mixture was centrifuged, dehydrated with successive washings in methyl alcohol, and finally dried over H_2SO_4 in a vacuum desiccator.

The three centrifugates were separately treated. The solubles in each were precipitated by adding an excess of methyl alcohol, settling overnight, decanting, and taking up the precipitate in methyl alcohol.

The yield of the four products was distributed as follows:

As less soluble	44.6%	
As more soluble	55.6	
a. From first centrifugate		47.4%
b. From wash of insolubles		7.8
c. From reprecipitation		0.4

For the tests described in the text, only the soluble fraction (*a*) was used, the others (*b* and *c*) appearing to be less pure, particularly the (*c*) fraction.

Preparation of whole starch triacetate: Powdered corn starch was carefully dried to between 2% to 3% moisture. To each gram of starch in a flask with agitator and condenser, 0.12 g of fused sodium acetate and 3.3 g of cp acetic anhydride were added quickly and the mixture stirred under anhydrous conditions. Heat was gradually applied and the mixture refluxed for 4 days with continued agitation until the mixture became more or less homogeneous. However, even with prolonged refluxing using larger quantities of acetic anhydride the solution never became perfectly clear.

The mixture was then run slowly into a large volume of water with vigorous agitation, regulating the pH, if necessary, from time to time within the range of 4.5 to 5.0. After several washings of the product, it was pulverized, washing was continued until the washings were substantially free from acetate ion, and it was then air dried. Acetyl found: 43.5%.

Separation of starch components by fractionally precipitating them as acetates: Twenty grams of whole starch triacetate were suspended in 600 ml of chloroform in a closed vessel and allowed to stand with intermittent stirring for about 10 days. Thereupon, 600 ml of petroleum ether was gradually stirred in and the mixture centrifuged in closed bottles. The centrifugate was decanted and the residue was

washed by suspending in a mixture of 100 ml of chloroform plus 100 ml of petroleum ether and recentrifuging. The residue was further purified by stirring up with 400 ml of chloroform, adding 300 ml of petroleum ether and centrifuging. The residue was then washed with petroleum ether until substantially free from chloroform and then dried.

The washings from the above were evaporated and added to the first centrifugate to which was then added with constant agitation 200 ml more petroleum ether and the precipitate was centrifuged out. The insolubles were washed with a small amount of a chloroform-petroleum ether solution (3 : 4), then with petroleum ether until chloroform free, and then dried. To the centrifugate an addition of 300 ml more petroleum ether brought down substantially the balance of the original acetate, which was washed with petroleum ether in the centrifuge and then dried.

Conclusions

Refinements in procedures are given for the estimation of amylopectin in corn, tapioca, and potato starches, from which it is shown that the percentages calculated from diastatic conversion are not as constant for these three types of starch as has been reported by earlier investigators.

The material not saccharified by diastase cannot be accounted for solely as gamma-amylose, from which it is concluded that the latter is not identical with amylopectin.

The more common procedures for separating amylose are reviewed, but in no case was a product obtained which converted straightforwardly and quantitatively to maltose with beta-amylase.

The products from two newer methods of fractionating starch are reported but none of these fractions answers the requirements for either amylopectin or amylose.

The concept of amylopectin and amylose, given by Maquenne, is still apparently hypothetical, and until it can be demonstrated, caution is advised in the use of these terms for designating various fractions of starch.

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QUANTITATIVE CONTROL TEST FOR IRON ADDED TO FLOUR

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(Read at the Annual Meeting, May 1941)

The control of the addition of iron to flour is important because it, in turn, implies control of the distribution of concentrate and added vitamin in enriched flour. The following method for the estimation of added iron in enriched flour is recommended for control work, where an accuracy within 0.5 to 1 ppm is considered satisfactory. Its application is limited to ferric iron compounds soluble in dilute HCl acid. The test is based on a colorimetric procedure, in which use is made of a series of five carefully prepared standards. Its accuracy depends on certain limits of added iron. For this reason the conditions of the tests have been chosen so as to conform to the prevailing practice, which calls for the addition of approximately 10 ppm. In the preparation of the standards, two distinct methods may be employed.

First Method

Prepare a series of flour standards using, as a base, flour of the same milling grade and mixture as the sample or samples to be tested and providing the following five stock standards: 120%, 110%, 100%, 90%, and 80% of the regular iron treatment.

These standard samples are most conveniently made by first preparing 2,000 g of flour containing 120% of the normal iron treatment. Then, by dilution of 400-g portions of this flour with the calculated amount of untreated flour, the other four flour standards can be obtained.

Apparatus

2 dozen small test tubes 4" × ½"
50-ml Griffin beakers
10-ml pipettes
2-ml pipettes
1-ml pipette in 0.1-ml divisions
Stirring rods

Reagents

Dilute HCl: 1,000 ml concentrated HCl (sp gr 1.18) diluted with 1500 ml distilled water.

Potassium ferrocyanide solution: 20 g potassium ferrocyanide in 100 ml distilled water.

Procedure: Four g of the five standard samples, respectively, are transferred to the 50-ml beakers, also 4-g portions of the sample or samples which are to be tested. Then to each beaker 10 ml of distilled water is added, and the flour and water are stirred together until a smooth suspension, entirely free of lumps, is obtained. To these suspensions, 10 ml of dilute HCl acid is added, and the contents of the beakers are properly stirred, left to stand for a few minutes, and then stirred again. After standing for about 45 minutes, enough supernatant liquid is available for the withdrawal of 2-ml portions.

These 2-ml portions are transferred to test tubes to each of which 0.1 ml of the potassium ferrocyanide solution has already been added. The tubes are shaken and then left to stand for about 30 minutes, when the color comparison of the unknown sample or samples with the standards may be made. If a centrifuge is available, which employs 50-ml tubes, much time can be saved by transferring the 4-g flour samples directly to the centrifuge tubes instead of the beakers. Centrifuging the suspension will naturally produce a much quicker and more complete separation of the liquid and solid phases of the suspension.

Second Method

The apparatus is the same as for the first method with the addition of a 2,000-ml volumetric flask, and the procedure is a modification of

the first method. Instead of a series of five flour standards, five stock samples of iron solutions in dilute acid are kept for reference standards. This has the advantage that it avoids a possible mixing error in the preparation of the standard flour samples.

These standard iron solutions are made up as follows: Calculate the weight of the iron compound to be dissolved in 2,000 ml of dilute acid on the following basis: 10 ml of this solution must contain the equivalent of 120% of the intended amount of added iron in 4 g of the enriched flour. Transfer this calculated amount of iron compound to a 2,000-ml flask, then add 800 ml of concentrated HCl acid, fill to the 2,000-ml mark with distilled water, let cool to room temperature, again fill to mark, and finally mix thoroughly.

Measure exactly 400 ml of this solution and transfer to a 500-ml Erlenmeyer flask. This portion is set aside as 120%. Again measure exactly 400 ml, transfer to a 500-ml Erlenmeyer flask, and add 36.5 ml of dilute HCl (1 to 1.5). Thoroughly mix. This is set aside as 110%. Again, 400 ml is measured and transferred to a 500-ml flask. To this 80 ml of dilute acid is added, thoroughly mixed, and set aside as 100%. The next 400 ml is transferred to a flask or beaker of a capacity considerably larger than 500 ml, and 133.3 ml of dilute acid is added and thoroughly mixed. This is the 90% standard. The fifth and last 400 ml is diluted and mixed with 200 ml of dilute HCl. This is the 80% standard.

It is evident that these standards must be kept free from evaporation or contamination.

Method of procedure: Weigh five 4-g portions of a flour (not enriched) of the same milling grade and mixture as the sample or samples to be tested for added iron. Transfer to beakers as above; or if a centrifuge is at hand, transfer directly to the 50-ml centrifuge tubes. The same procedure is followed with the 4-g portion, or portions, of the enriched samples to be tested.

To each of the beakers or centrifuge tubes, 10 ml of water is added; flour and water are thoroughly stirred with a stirring rod until smooth. Add to the five suspensions, representing the unenriched flour, 10 ml of the five standard iron solutions—120%, 110%, 100%, 90%, and 80% respectively. To the suspensions of the enriched sample or samples which are to be tested, 10 ml of dilute acid is added. They are stirred, left to stand for a few minutes, and then stirred again, after which they can be either centrifuged or left to settle out for about 45 minutes. Two-ml portions of the supernatant liquid are transferred to the small test tubes, to each of which has previously been added 0.1 ml of potassium ferrocyanide solution. The tubes are thoroughly shaken, to insure complete mixing, and are then set aside for about

half an hour, after which color comparisons can be made. It is best to make comparisons with reflected light.

Remarks

As already mentioned, the sensitivity of the test depends on certain limits of iron concentration. Under the conditions of the present methods of procedure, it was found that a 4-g flour sample is well adapted for prevailing conditions of iron fortification (approximately 10 ppm). If, at some future date, the present level should be changed, it will be advisable to change the size of the flour sample accordingly. If, for instance, the iron dosage should be doubled, only 2 g instead of 4 g must be used as the flour sample in this method.

A convenient way to clean the test tubes is to rinse them with concentrated KOH solution, then with water, followed by dilute HCl, tapwater, and finally, distilled water.

It is desirable that the test tubes used for this colorimetric procedure be carefully matched as to uniformity.

The change of the base flour at any change in the milling mixture involves the preparation of new standards in the case of the first method, but not in the second method. The second method therefore deserves preference.

The change of the base flour at every change in the milling mixture is necessary because of the variation in the ferric iron content of different wheats and their milling products.

THE RELATION OF FATS TO TEXTURE, CRUMB, AND VOLUME OF BREAD

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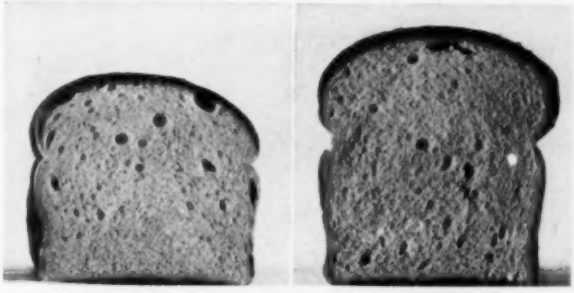
(Read at the Annual Meeting, May 1941)

The results accomplished by the use of fats in bread making are a change in flavor, generally considered desirable; a more tender crumb; and in most instances a noticeable improvement in the quality of the bread when judged by texture and loaf volume. Comparatively little research on the action of shortening in bread making has been recorded during the past twenty years. Platt and Fleming (1923) reviewed and interpreted the action of shortening in bread making. Fisher and Jones (1932) studied and described effects of various shortenings. Heald (1937) reported the effects of varied amounts of several commercial shortenings. Bohn and Bailey (1937) measured the action of

shortenings on the physical properties of bread doughs. Sullivan (1940) studied and recorded the effects of substituting various shortenings for the natural flour fats in extracted flour. It is the purpose of this discussion to consider only the effects of fats upon the texture and loaf volume of the finished bread.



No shortening 1% commercial shortening

Fig. 1. Effects of shortening on bread.

Figure 1 illustrates the effects produced by 1% of commercial semisolid shortening when used in dough that is matured to its optimal baking point with sodium chlorite and baked with the no-time method. The main difference produced by the use of the shortening is marked increase in volume with somewhat more open texture. This type of result is typical of the effects of shortening, though the no-time method of baking intensifies the differences. Heald (1937) found that all classes of shortening, 2% to 4%, increased the loaf volume of the bread.

In this paper the no-time method described by Baker and Mize (1941) has been used throughout. However, the technique there described has been changed by using a commercial molder in place of an extruder. To obtain the best results it is necessary to mold such doughs immediately at the end of the mixing period. This eliminates many variables that are present in other methods of baking and thus simplifies the interpretation of results.

Figure 2 shows no-time bread in which 3% of fat is used in liquid and also semisolid form. The increased amount of semisolid fat has produced an improvement in texture over that obtained by the 1%. However, when the liquid fat was used in this dough some loss in volume and noticeable deterioration of texture occurred. Apparently there is an effect caused by the physical state of the fat. This difference produced by fats was noted by us in 1939 and is also observed in baking straight 2½-hour doughs. Results similar to those produced by liquid fat were obtained with many liquid shortenings such as salad oil, cottonseed oil and peanut oil. Results comparable to those with the

semisolid fats have been obtained with all other commercial shortenings such as lard, cocoa butter, tallow, commercial hydrogenated shortening, etc. An improvement was invariably obtained in the quality of bread baked when such semisolid fats were used.

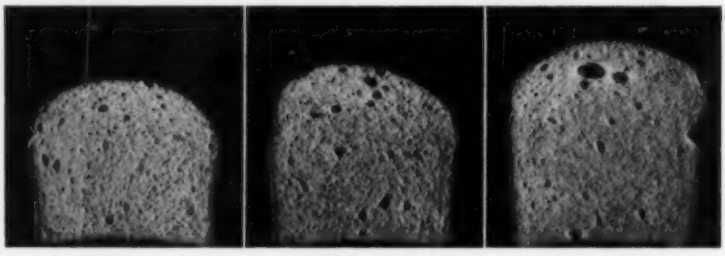
These observations led us to test the effect of hard, brittle fats in bread making. Such nonplastic fats are not available in nature but by complete hydrogenation fats can be made so hard they can readily

No fat 3% liquid fat 3% semisolid fat

Fig. 2. Typical effects of liquid and semisolid fats on no-time bread.

be ground in a mortar. Completely hydrogenated cottonseed oil was ground and sieved to several mesh sizes up to 100 mesh and mixed in the doughs during the entire mixing period. The finer the mesh used the better the result obtained.

Usually greater improvement in the quality of bread was obtained by the use of this brittle, hard fat than with commercial shortenings. It gave increased volume and finer texture, as shown in Figure 3.



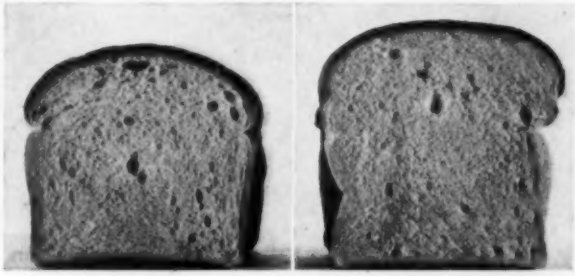
No fat 0.2% hard fat 1.0% hard fat

Fig. 3. Effects of hard fat on no-time doughs.

It is apparent that 0.2% has given some improvement and 1% has given marked volume and texture improvement. Comparing the effect of 1% of hard fat in Figure 3 with 1% of commercial shortening in Figure 1 it is noticeable that the hard fat has produced better results. Other hard substances such as carnauba wax, hard Russian paraffin, and Chinese insect wax, were ground to fine powder and tested in a

manner similar to that shown above. These materials gave an improvement in bread volume and texture similar to that of the completely hydrogenated cottonseed oil.

Figure 4 shows the results obtained by dissolving a small amount of hard fat in a liquid fat. Two percent of completely hydrogenated fat was dissolved in linseed oil. This was more than the minimum required to give a cloudy appearance in the oil. The total amount of hard fat added here amounts to only 0.06% calculated on the amount of flour used in the dough. Apparently this small amount of hard fat dissolved in the liquid fat exceeded its solubility when cooled, as shown by the cloudiness, and thus, due to fineness of the fat crystal, was able



3% linseed oil 3% linseed containing 2% hydrogenated cottonseed

Fig. 4. Effects of solid fat blended with liquid fat.

to produce a noticeable effect with a smaller amount of hard fat than in any of our other experiments.

It is apparent that the improving action of such hard powders cannot be due to the often cited lubricating property of shortenings. A common explanation of the improving action of shortening is that it lubricates the fibers or filaments in the dough so that they slip by each other and better cell structure is thereby obtained. Certainly this explanation is not applicable to the results obtained from these hard powders. Neither is it satisfactory in explaining the results obtained by either semisolid fats or the fluid fats, for were the explanation true fluid fats should give results approaching those of semisolid fats since their lubricating properties are very pronounced.

Cocoanut oil has a sharp melting point slightly below the temperature at which doughs are ordinarily handled. This enabled us to test the effect of the physical state of a fat on doughs by mixing and proofing at temperatures above and below the melting point of the fat. The cocoanut oil used here had a melting point of 78°F and a solidifying point of 71°F. We therefore mixed two series of doughs, one at 80°F and the other at 70°F, and maintained these temperatures for each dough throughout the mixing and proofing period.

Figure 5 gives the result obtained from this experiment. These results show that when the bread was mixed and proofed at 70°F it made little difference whether cocoanut oil was added to the dough as a solid or a liquid. Substantially the same improvement was obtained. In contrast to this, cottonseed oil when mixed at this temperature gave a very poor loaf. However, when doughs were mixed at 80°F no improvement in volume was obtained by the use of cocoanut oil in either solid or liquid form though the solid form gave some improvement in texture. This was doubtless due to the small difference between the melting point of this fat and the temperature of mixing so that a

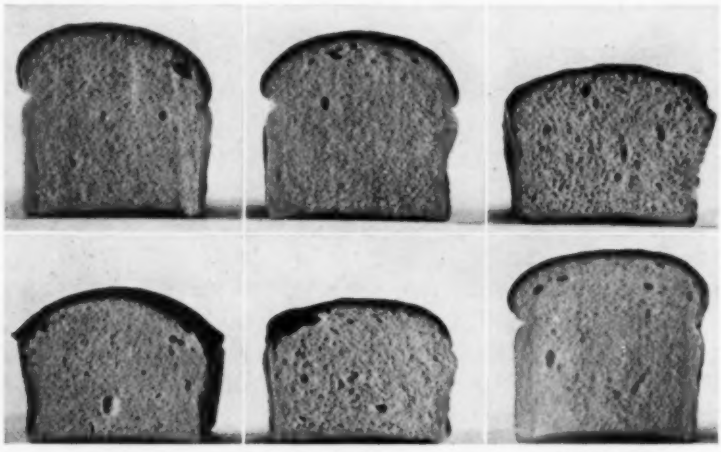


Fig. 5. Relation of dough temperature to effect of cocoanut oil in bread making.

considerable period may have elapsed before the solid fat became liquefied during the mixing, thus producing some of the effects of a solid fat. However, when cocoanut oil was added in a liquid form results very similar to that with cottonseed oil shown above were obtained. Commercial shortening was used at this higher temperature, showing that semisolid fat which does not liquefy at 80°F. produced good results at this temperature. This experiment proves that the effects obtained from a given fat are largely determined by whether it is a solid or a liquid at the temperature of the mixing and proofing.

In an attempt to find out something about the changes that occur in fat when mixed with dough, gluten was washed from such dough at the completion of the mixing period, before panning. Such doughs were carefully washed in 3% sodium chloride at the same temperature

as the dough at the end of the mixing. Thus no change in the salt balance of the ingredients was encountered during the washing.

In all cases where liquid fats were employed very little fat was recovered during the washing process. Substantially all the fat remained in the gluten except a very small amount which was held in colloidal suspension in the wash water. By centrifuging the wash water this fat could be separated.

When doughs made with semisolid fats are washed, the gluten retains the major portion of the fat. However, a considerable amount separates and floats on the wash water and can be skimmed off and collected. When solid fats are used, similarly a considerable portion remains in the gluten but another considerable portion floats on the wash water in its original condition and can be recovered.

TABLE I

EFFECT OF TEMPERATURE ON THE BLENDING OF COCOANUT FAT WITH FLOUR FAT DURING MIXING

Three percent (10.2 g) cocoanut fat used per loaf.

Mixing temperature	Washing temperature	Amount fat recovered	Iodine number
85°F	65°F	0.156 g	26.0
65°F	65°F	5.6 g	15.2

Theoretical Calculations

10.2 g cocoanut fat	I_2 No. 9.5
3.4 g flour fat	I_2 No. 110.0
Blended 13.6 g mixed fat	I_2 No. 34.6

Table I shows the effect of temperature on the blending of cocoanut fat and flour fat during dough mixing. Two doughs were mixed at different temperatures, one above the melting point of cocoanut oil and the other below. After the mixing, the warmer dough was cooled to the temperature of the cooler one. The gluten was then washed at the lower temperature from each dough. The weight and iodine number of the fat skimmed from the surface of the gluten wash solutions were determined.

Three percent (10.2 g) of cocoanut fat was used; the flour contained 1% (3.4 g) of natural fat. If all of the fat in the dough had been recovered on the surface of the gluten wash solution, 13.6 g of fat would have been obtained. When the dough was mixed above the melting point of the cocoanut fat very little was recovered, indicating that the fluid oil had completely blended with the dough and was all retained in the gluten with the flour fat. One might assume that the cocoanut fat and the flour fat had blended at this temperature of mixing. That this was the case is indicated by the high iodine number of 26 in the small

amount of fat recovered. Cocoanut oil has an iodine number of 9.5. A mixture should have a theoretical iodine number of 34.6. When the dough was mixed below the melting point of the cocoanut oil, at 65°F, incomplete blending of the fats was indicated by the iodine number of 15.2 and by the large amount of fat which floated on the gluten wash water. These figures indicate that a considerable proportion of the semisolid fat blended with the flour fat as shown by the iodine number of that portion recovered. A much larger proportion has been retained in the gluten and doubtless is still more closely blended with the flour fat.

These experiments suggest that the effects, which account for the improvement in bread obtained by the use of fats, are due to the blending of the added fat with the natural oil of the flour, thus producing changes in the properties of this natural oil. Most liquid fats, though they blend completely, are incapable of raising the melting point or increasing the viscosity of the flour fats. They blend together but still remain fluid. Semisolid fats, on the other hand, are capable of blending with the flour fat and raising its melting point and increasing its viscosity.

Castor oil acts differently from other liquid fats. Fine improvement of bread quality is obtained by its use, giving results very similar to the effects of commercial shortening.[1] This oil has a very high viscosity which does not drop as rapidly with increasing temperatures as does the viscosity of other oils. These characteristics suggest that castor oil operates in the same manner as other fats that improve bread quality—namely, by changing the character of the natural fats in the bread film, which by their fluid properties when heated permit gases to pass through the films. It may be that the highly viscous castor oil itself plugs these openings, thereby preventing porosity.

In a previous article (1939, p. 693) we published a chart that shows clearly the effects of shortening on optimum oxidized dough. In the test penetrability, pressure, and volume of dough while being baked in the electric oven were correlated and show that all three doughs, *i.e.* with no fat, with liquid fat, and with semisolid fat, have the same penetrable characteristics. Only the dough containing the semisolid fat shows the characteristic increase in pressure which accompanies increasing loaf volume, as contrasted to the others containing liquid fat or no fat. At that time we said "One is led to speculate as to why this should be. The fine texture of all of these loaves shows that there was no coalescence, nor was the cell structure disrupted as was shown by their final ability to retain gas. Apparently gas must escape from these doughs during the softening period by diffusion through the cell

[1] E. A. Fisher and C. R. Jones in 1932 observed this improving action of castor oil.

walls; hence the difference in their ability to retain gas may be a difference of cell-wall porosity. Here is a clew: solid [semisolid or hard] shortening may prevent cell-wall porosity; whereas fluid shortening or no shortening is unable to do so." We believe this observation is further proved by our present work.

In a paper by Baker (1939) a statement was made (p. 732) that semisolid shortenings increase the permeability of bread by air. Furthermore, as stated on page 733, "Breads from some small bakeries are usually highly permeable, excepting those made without the use of shortening, in which case they are generally fairly impermeable even when of very coarse texture. Apparently shortening has an effect which prevents the cellular structure from finally closing or sealing the pores. This is directly opposite to the action of shortening during the earlier stages of baking, as shown in a previous paper."

TABLE II

EFFECTS OF FATS ON AIR PERMEABILITY OF BREAD
Matured no-time dough.

	Permeability	Volume	Texture
		ml	
3% commercial shortening	43	2,640	102
1% commercial shortening	34	2,470	102
1% completely hydrogenated cottonseed	34	2,440	103
3% cottonseed oil	11	1,970	100
No fat	9	2,060	103
3% mineral oil	48	1,800	97

We have further investigated this permeable property of bread in connection with certain shortenings as shown in Table II. It has been noted that commercial shortenings and hard, completely hydrogenated fat produce bread of high permeability, whereas cottonseed oil or no shortening gives fairly impermeable bread. Permeability of bread by air is due to the rupture of the cells during the latter stages of expansion of the loaf after starch swelling has made the dough less fluid by taking up water. When the fluid properties of the dough mass are so decreased that the cells can no longer extend themselves and remain intact, the cell wall must rupture to relieve the pressure. The permeability to air of the resulting baked bread is thus increased. Apparently where no shortening is used or liquid fat added, the cell structure, during heating, becomes so porous at the points where these liquid fats are located that the gases escape without breaking the cell wall and the resulting bread has low permeability by air. In contrast, when regular shortenings or hard fats are used, the cells become less porous, the escape of gas is prevented, and the force breaks the cell wall. Possibly this breaking of the cell wall in the latter case is helped

by the incomplete homogeneous incorporation of the semisolid fats indicated by the gluten washing experiments. The fats may lie across the cell wall and promote the breaking of the cell.

The effect of increasing the permeability by mineral oil in Table II supports the latter hypothesis, for here the oil is a liquid which does not blend readily with any of the natural oils in the dough, thus being forced to find some other location. This doubtless would lead to points of weakness in the cell wall and disruption of the cell. This mineral oil bread, which has the smallest volume, actually has the greatest permeability of those tested.

Although we have as yet made no detailed studies, it is apparent to all observers in this laboratory that breads which show high air permeability can always be rated tender breads and those which are impervious to air are more tough in character. This indicates that increased tenderness in bread, where the composition remains the same, is due to the disruption of the cell wall by the gas pressure in the baking process. The greater this cell wall breakage, the more tender the bread.

Discussion and Summary

In 1939 we observed that though semisolid fats all gave the usual improvement in bread quality, liquid fats did not when used in the same manner. Our experiments indicated that doughs containing liquid fats or those containing no shortening, when heated, begin their oven spring at exactly the same rate as doughs containing semisolid fats. However, the oven spring and the pressure within these first two doughs ceases abruptly. No difference in the physical properties of these three doughs could be shown. Therefore, this sudden increase in porosity of dough which semisolid fats prevent seems to be related to some difference in the properties of the solid and liquid fats themselves.

Semisolid fats are not the only fats that improve bread quality. Hard fats which possess substantially no plastic properties will, when finely pulverized and mixed with the dough through the entire mixing period, produce improvement similar or superior to that of commercial shortenings. Furthermore, when a liquid fat is supersaturated with a hard fat so that the mixture becomes cloudy, marked improvement in the baking quality of the bread is obtained over the use of liquid fats containing lesser amounts of dissolved hard fats.

When high-viscosity fats, such as castor oil, which maintain their viscosity during heating, are used, baking improvement is obtained. These observations suggest that those fats which improve baking quality have the property of plugging the pores responsible for the leakage in the cell walls. That this is the case was further indicated

by experiments with cocoanut oil. Bread doughs mixed and proofed below and above the melting point of cocoanut oil gave results that corresponded in all respects to those obtained with semisolid fats on the one hand and liquid fats on the other, thus proving that the physical state of the fat is the determining characteristic. Separation of fats from doughs during gluten washing indicated that the added fats mingle with the natural fats in the flour, thus further indicating that the effects obtained by fats in bread making are associated with the admixture of the added fat to the natural fat.

It may be argued, in the case of semisolid fats, that improved effects obtained are due to the liquid flour fat being moved out of location in the dough and taken up by the semisolid fat, thus preventing the leaks. This argument appears to us to lack validity because the removal of the fat would not remove the hole from the dough film. Also this removal theory is not supported by the character of hard fats recovered by gluten washing. These fats were only slightly wetted by the natural fats. Furthermore, in gluten washing when dough is made with liquid fats a very small amount of the liquid fat is recovered. It possesses a high iodine point, indicating nearly complete blending of the liquid fat with the natural fat of the flour and retained there as shown by its presence in the gluten. It is difficult to explain the effects obtained by a supersaturated solution of solid fat and liquid fat or the effects of castor oil by the removal theory. This theory has therefore been discarded by us.

Semisolid and hard fats produce a bread texture which is highly permeable by air, indicating that the cell structure is largely disrupted. Such breads are always more tender. These results can be explained by the assumption that such fats are not completely emulsed and blended with the natural fats in the dough and remain in masses which weaken the dough film in many spots. This characteristic of semisolid fats may produce the changes in dough consistency which lead many workers to consider the effects of fats as one of lubrication. The lubrication theory, however, is untenable in view of the effects of hard fats and the lack of effect of liquid fats which are themselves good lubricants.

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CHEMICAL FACTORS AFFECTING THE BAKING QUALITY OF DRY MILK SOLIDS. I. CORRELATION OF pH AND BAKING SCORE [1]

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Within the past 12 years the use of dry milk solids in the baking industry has increased nearly 300% as shown by the American Dry Milk Institute (1939). In spite of this increase it is regrettable that not all dry milk solids are of uniform good quality and can be used for baking. To offset this difficulty baking tests have to be made on samples of each batch to prevent a poor baking product from being sold.

The reasons for the variability in the baking quality of dry milk solids is not known, although many properties of the dough mixture and of the dry milk solids have been investigated. These investigations do not cover what would appear to be a necessary preliminary study of the problem, namely, whether or not the milk before processing had undergone fermentation. Such fermentation would be expected to change the pH of the dry milk solids from the natural narrow limits of normal milk as indicated by Caulfield and Riddell (1936) and Lisk (1924). Furthermore, if fermentation should play a part in this defect it is to be expected that it would be of a seasonal nature.

This study was rendered possible by the cooperation with us of the Consolidated Dairy Products Company of Seattle who maintain a baking laboratory to test the dry milk solids they sell from their cooperating manufacturing plants. Thus the samples supplied by them

[1] Scientific Paper No. 488, College of Agriculture and Agricultural Experiment Station, State College of Washington.

[2] American Dry Milk Institute Research Grant, 1938 to 1940. A part of the data used here were submitted by G. H. Farrah in partial fulfillment of the work required for the degree of Master of Science, 1940.

were of known baking score and represent dry milk solids manufactured between June 9, 1939, and October 15, 1940. With a few exceptions heat treatment of 180° to 190°F for one-half hour, followed by a spray process of drying, was used in their manufacture. There were 3,170 samples in all.

The object of the work reported in this paper was to determine whether there is any correlation between the normal pH of dry milk solids and their baking score, and also to find whether the variations in pH and baking score are seasonal in nature.

Methods

The experimental baking and scoring of the loaves were done by the Consolidated Dairy Products Company at Seattle. They used a standard baking formula and the loaves were compared with a control baked at the same time and scored at 89.75. The instrument used for the pH determinations was a Beckman glass electrode pH meter laboratory model G. This instrument was found to be very satisfactory.

In making the pH determinations 10 one-gram samples of dry milk solids were weighed into test tubes and each shaken for 30 seconds with 9 ml of distilled water. Between 5 and 10 minutes were allowed for hydration of each sample. Because of the evidence of protein film formation on the glass electrode as noted by Parks and Barnes (1935), the electrode was checked against a standard potassium acid phthalate buffer after every fifth sample. The pH values were reproducible to 0.02 pH units.

The samples were weighed on a Torsion Dairy Scale with a sensitivity of 0.01 g. The effect of errors in weighing was determined and found negligible. The effect of differences in the periods of time allowed for hydration of the samples was also investigated. Samples of dry milk solids were reconstituted with water at 9°C. A 5-ml portion was immediately warmed to 24°C and its pH determined. The remainder of the sample was kept at 7°C in order to prevent bacterial action. At various time intervals the pH of the mixture was determined in a similar manner. During the first 15 minutes there was no change. After one hour the average pH had increased about 0.03 unit. After five hours it had increased 0.05 to 0.10 unit.

Experimental Results

The frequency distribution for pH is given in Figure 1. The mean pH of all samples was $6.476 \pm .00080$. The distribution about the mean is approximately normal as shown by the figure. The distribution of baking scores, also shown in Figure 1, is not normal because

most of the samples fell close to the upper limit of the baking score range. The mean baking score was 89.17±.0078. About 80% of all the samples had baking scores between 89.00 and 89.75. A comparison of the variability of the two measurements can be had from their coefficients of variation, which for pH is 1.03% and for baking score 0.73%.

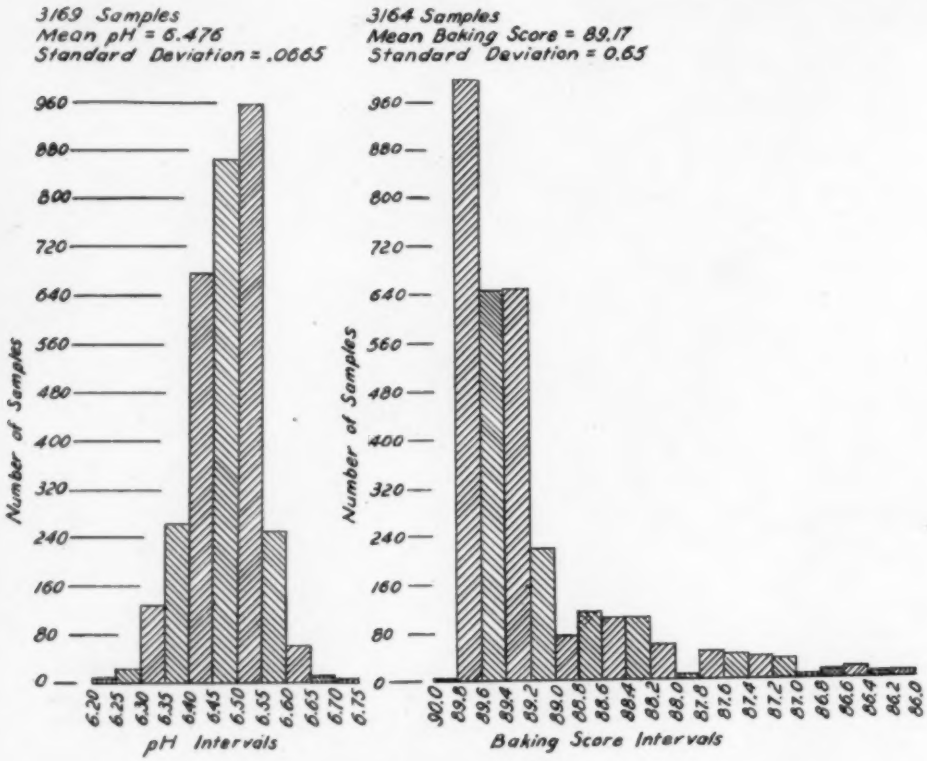

Fig. 1. Distributions of pH and baking scores.

One of the factors thought to have a possible influence on the baking score and pH of the dry milk solids is the geographical location of the dairies from which the milk originated. No very extensive study of this problem could be made, however, because all the samples placed at our disposal came from plants located in northwestern Washington except those coming from a plant in southwestern Idaho, flume No. 7. Table I shows for each flume (unit of operation in the manufacturing plant) the mean pH and baking score together with their probable errors. There seems to be a definite association of low baking scores with high pH values. The curve secured is very similar to that of Figure 2.

TABLE I

AVERAGE BAKING SCORE AND pH FOR EACH FLUME

Flume number	Approximate location	No. of samples	Average pH	Average baking score
2	N. W. Wash.	741	6.480±.0016	89.20±.016
3	N. W. Wash.	337	6.484±.0021	89.11±.021
5	N. W. Wash.	600	6.515±.0014	89.04±.021
6	N. W. Wash.	731	6.445±.0013	89.20±.017
7	S. W. Idaho	728	6.468±.0018	89.26±.012

Figure 2 shows for all our data baking score plotted against the pH of the dry milk solids used in the baking. The numerals beside each point indicate the number of samples averaged. The trend seems to be slightly upward between the pH limits of 6.32 and 6.47. However, when treated statistically this was found not to be significant. In other words, the pH of the dry milk solids, when between 6.32 and 6.47, had no effect on their baking score. When the pH limits were extended beyond 6.47 (practically the mean value) a very significant lowering of the average baking score was observed.

To show more clearly the significance of this point, Table II was prepared. It divides the data into six baking-score groups and shows the percentages of the various groups that have a pH above the mean.

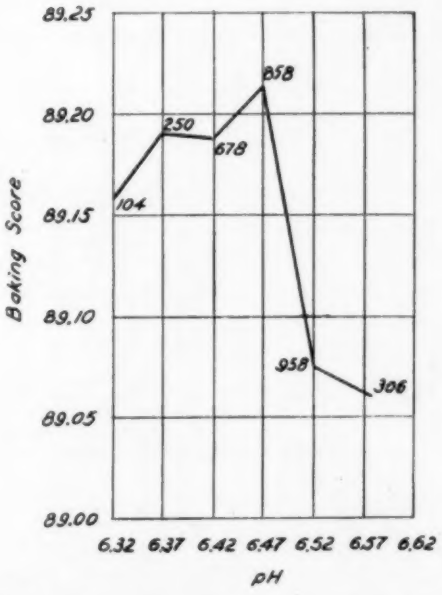

Fig. 2. Correlation of the baking score with pH. The numbers of samples averaged are indicated at each point.

TABLE II

PERCENTAGE OF SAMPLES WITH pH ABOVE 6.5

Baking score	Total number of samples	Number with pH above 6.5	Percent above pH 6.5
Above 89.5	998	449	44.8
89.0 to 89.5	1521	506	33.2
88.5 to 88.9	195	86	44.1
88.0 to 88.5	250	109	43.6
87.0 to 87.9	162	77	47.5
Below 87.0	43	37	86.1

In the group having baking scores below 87.00 nearly twice as great a proportion had pH values above 6.50 as any other group.

A distinct trend of pH with the season of the year is shown in Figure 3. The average pH values for 20-day intervals were calculated and plotted against time. During the months of July, August, and

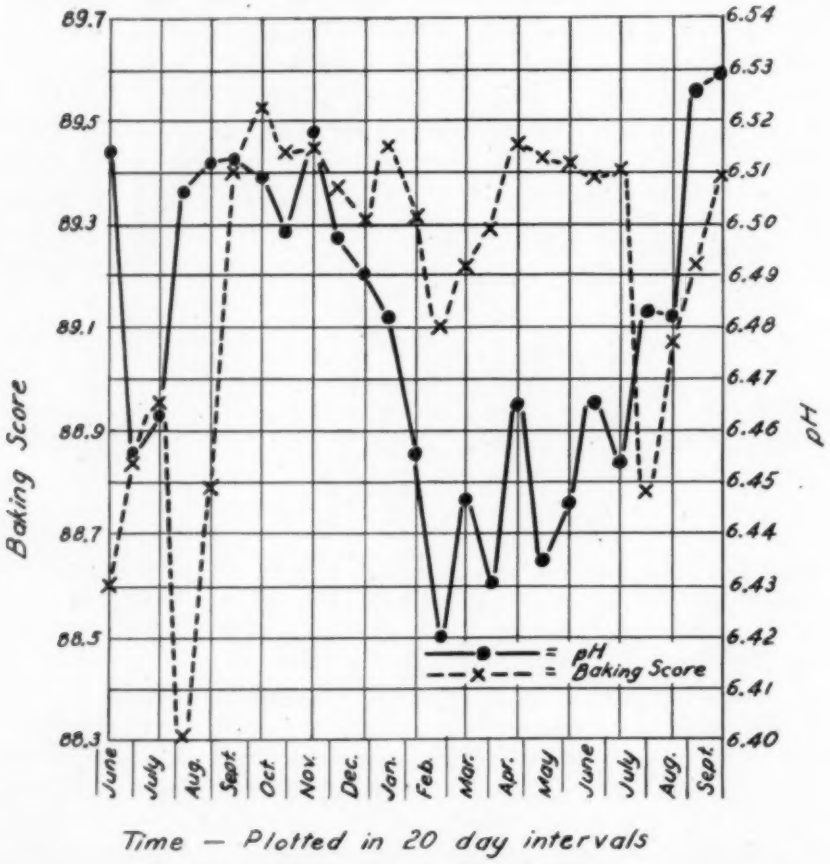

Fig. 3. Seasonal variations of pH and baking score.

TABLE III

CORRELATION COEFFICIENTS OF BAKING SCORE WITH pH

Date limits of manufacture of powder	pH limits 6.00–6.49		pH limits 6.45–6.79	
	Number of samples	r_{xy}	Number of samples	r_{xy}
1939				
June 9–June 28	23	+.135	83	−.134
June 29–July 18	35	+.250	42	−.460†
July 19–Aug. 7	56	+.034	62	−.024
Aug. 8–Aug. 27	44	−.049	98	−.201*
Aug. 28–Sept. 16	47	−.004	138	+.849†
Sept. 17–Oct. 6	64	+.341†	135	−.116
Oct. 7–Oct. 26	61	−.202	115	−.196*
Oct. 27–Nov. 15	69	−.052	117	+.050
Nov. 16–Dec. 5	47	−.122	128	−.005
Dec. 6–Dec. 25	70	+.060	123	−.082
Dec. 26–Jan. 9	34	−.297	53	+.128
1940				
Jan. 10–Jan. 29	87	+.077	101	−.057
Jan. 30–Feb. 18	113	−.041	73	+.318†
Feb. 19–Mar. 9	128	−.080	24	+.118
Mar. 10–Mar. 29	120	−.073	62	+.129
Mar. 30–Apr. 18	116	+.080	39	−.154
Apr. 19–May 8	117	+.074	101	−.243*
May 9–May 28	122	+.065	68	−.152
May 29–June 17	122	+.073	74	−.510†
June 18–July 7	120	−.002	114	+.068
July 8–July 27	135	−.064	114	+.126
July 28–Aug. 16	116	+.118	139	−.136
Aug. 17–Sept. 5	28	−.099	32	+.310
Sept. 6–Sept. 25	14	−.249	40	+.289
Sept. 26–Oct. 15	5	+.129	30	+.239
June 9–Aug. 27, 1939	—	—	285	−.205†
Mar. 30–June 17, 1940	—	—	282	−.265†

* Correlation coefficient significant (5%).
† Correlation coefficient highly significant (1%).

September, 1939, there was an increase in the average pH which reached a maximum of 6.51 in October. From then on there was a sharp decline to an average of 6.43 by the first of March, followed by a gradual increase during the spring and summer. These various trends have been tested statistically and found significant.

In a similar manner the averages of the baking scores for 20-day intervals were calculated, plotted against time and the significance of the various trends determined statistically. The downward trend at the start was found to be quite significant (June, July, and August, 1939). Then there was an abrupt rise during September which took the average baking score from a low of 88.3 to a high of 89.5 in October, 1939. Such a high average shows that there were practically no poor baking samples manufactured during that period. During the winter months there was a gradual, but significant, downward trend to an average of 89.1 in February, 1940. The average baking score then

took a sharp upward turn, leveling off at an average score of a little above 89.4 during April, May, June, and July. For the period July 28 to August 16, 1940, the average baking score had dropped to 88.8. This was followed by a rise during September. Thus in both years poor baking quality was associated with the hottest part of the year at which time the pH was above the average and rising.

A comparison of the curves in Figure 3 suggests that there is no simple relationship between pH and baking score. Figure 2, however, shows a definite association between pH values and low baking scores. In order to get a more accurate picture of the relationship between pH and baking score the effect of season was eliminated. This was done by determining two correlation coefficients for each 20-day interval, one for the pH limits of 6.00 to 6.49 and the other for the pH limits of 6.45 to 6.79. The results are shown in Table III.

Most of the correlation coefficients in Table III are not significant. Those that are slightly significant are designated by a single asterisk; those that are highly significant are designated by a dagger. The algebraic mean of the correlation coefficients for all 25 groups is +.01201 for pH limits 6.00 to 6.49 and +.0051 for pH limits 6.45 to 6.79. Neither value is significant statistically.

Discussion

The pH of fresh milk from normal udders falls within the range of 6.22 to 6.77 as reported by Sommer and Matsen (1935). Our results obtained from the composites of many cows would be expected to show a much more narrow limit. The mean value of our 3,169 samples was found to be 6.476 (Table I). The calculated mean of the 386 samples reported by Sommer and Matsen was found to be 6.476. The identity of these means indicates that the tendency toward acid or alkaline fermentation, if it occurred in the milk used for dry milk solids, was very nicely balanced.

It is possible, but unlikely, that the pH values within the range studied will themselves affect the baking quality. St. John and Bailey (1929) found the buffering action of dry milk solids on bread doughs to be appreciable but they observed an accelerated fermentation rate due to the milk and concluded that there must be a factor in the milk which compensated for the decrease in acidity.

The direct correlation of pH with baking score for all the data shows that the more alkaline samples of milk are generally associated with the lower baking scores. Nevertheless when the effect of season was eliminated by determining the correlation coefficient for each 20-day period and averaging the resulting values, all significance was lost in the correlation between baking score and pH (Table III).

The effect of the season of the year on the pH of milk has not been

studied very extensively. According to Caulfield and Riddell (1936), the most important factor influencing the pH of normal milk is the stage of lactation. With the advance of lactation the pH increases to the alkaline side. It was not possible for us to study directly the effect of the stage of lactation on the baking quality of the milk because our samples represented composites of many animals.

The feed of the cow is a possible factor influencing the pH of the milk. The work of Sommer and Hart (1921) and others indicates that such an effect is unlikely, although the problem is not settled in the minds of many workers in the field.

In our work it is interesting to note that the minimum value for the pH (averaged in 20-day intervals) occurred in February and the maximum value occurred in August. If lactic-acid-forming bacteria were entirely responsible for the lower values, such values should occur in the warmer season.

Summary

An examination of 3,170 samples of dry milk solids for pH and baking score was made.

There was no significant effect of the pH of dry milk solids on the baking quality of the doughs made from them.

Dry milk solids with poor baking quality were produced in the main during the latter part of July and through August for both years studied. The pH at this time of the year was above average.

Acknowledgments

Thanks are tendered to the Consolidated Dairy Products Company, Seattle, and their staff for their unfailing cooperation in supplying the samples and baking scores of the same; to the American Dry Milk Institute, Chicago, for providing a large part of the funds for the work; also, to the Research Department of the Federal Land Bank, Spokane, who through the assistance of Dr. H. Ashley Weeks enabled the punch card system to be used for sorting the data.

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FACTORS WHICH INFLUENCE THE PHYSICAL PROPERTIES OF DOUGH. IV. THE EFFECTS OF SURFACE ACTIVE AGENTS ON THE CHARACTERISTICS OF THE CURVES MADE BY THE RECORDING DOUGH MIXER[1]

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Wide variations in dough development curves made by the recording dough mixer were reported by Swanson (1939). What factors cause these variations? Why is one flour wetted more rapidly by water than another? The steeper rise of the curve is apparently due to the more rapid wetting. Why is there a more apparent breakdown, shown by the steep downslope and narrowing of the curves of Chiefkan and Early Blackhull, than with the more rounded top and more gradual downslope of Turkey, Tenmarq, and the spring wheat flours? Why are some flours more sensitive to amount of mixing than others? That the amount of protein and absorption influence the pattern of the curves has been shown by Swanson (1941) and also by Larmour, Working, and Ofelt (1939).

It was thought that the use of surface-active agents, because of their known effects on the interfacial tension of water films, which might also manifest themselves in dough, would throw light on some of these questions. Before proceeding to the experimental evidence, some theoretical consideration on which the investigations were based will be given.

Gluten Structure in Dough

In a well mixed dough, the gluten seems to exist as a continuous three-dimensioned network (Swanson, 1925, 1937). It is probable that on mixing in the pull-fold-repull type of mixer such as the National-Swanson-Working recording mixer, the protein molecules assume the fibrillar type. In the dough which has been mixed just enough for the water to envelop all the flour particles, the protein particles which form the wet gluten are arranged in a heterogeneous pattern. With the pulling, folding, and repulling, the fibrillar protein molecules become oriented into a more or less parallel pattern, arranged as a three-dimensioned gluten network in which the starch and other substances are enmeshed. The increasing resistance to the moving pins, shown in the upward swing of the pen, is due to the gradual perfecting of the parallel pattern. The

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perfecting of this parallel pattern is shown in the increasing smoothness of the dough, the appearance of which may be taken as an indication of the probable optimum amount of mixing.

Water Films in Dough

In a well mixed dough, the water films which cover the starch granules and the strands of the three-dimensioned gluten mesh form a continuous phase. The water molecules next to the actual surfaces of the starch and gluten are so strongly adsorbed that they have no freedom. Layers of water molecules superimposed on these have more and more freedom as the distance from the surface of the solids increases until in the outer layers of the water films the freedom approaches that which exists in a body of water. It is this freedom of the water molecules in the outer portions of these films which allows the sliding of the gluten particles on each other and on the starch. The formation of a protein film over the starch granules has been suggested by Markley (1938). The thickness of the water films varies with the amount of absorption.[2] The more water used in absorption, the greater the freedom of the water in the outer layers and hence the less resistance to the pins which pull, fold, and repull the dough. For this reason the height of the curve will vary inversely with the amount of absorption water (Swanson, 1941). That both the quantity and quality of protein, as in Tenmarq and Chiefkan, influence the pattern of the curve was shown by Larmour, Working, and Ofelt (1939). The quantity of protein directly influences the density of the gluten mesh since with a flour of higher protein there is more material to form strands.

Effect of Interfacial Tension

The viscosity or internal friction of the water molecules in these liquid films will have an influence on the ease of sliding of the dough particles on each other and hence on the curve characteristics. The addition of substances that lessen the adhesional tension of the water for the gluten would influence the ease with which these gluten fibrils move over one another. Less strongly adsorbed water would allow greater freedom of movement, which would be manifest in a longer time of dough development and also a prolongation of the period of breakdown. Consequently the presence of substances that lower the interfacial tension between water, gluten, and starch should have a marked effect on the mixing behavior of the dough as shown in the curve characteristics.

[2] The term *absorption* will be used for reference to the amount of water used to mix a dough of desired consistency or as understood in the baking trade.

Surface-Active Agents

Many substances have been developed recently that have the property of greatly reducing the surface tension of water and becoming largely adsorbed at interfaces. These are commonly known as " wetting agents " and also as surface-active or interfacial-tension-reducing agents. Some agents such as alcohol and organic acids that would be included in this classification are unsuitable for use with flour because they have a disintegrating effect on the gluten molecules.

A survey of commercial products manufactured for various purposes, particularly those of hydrophilic nature, revealed a large number suitable for this investigation. It was first thought that the main effect of these surface-active agents would be on the interfacial tension of the water films in the dough. It was soon found that their action was not limited to the reduction of the interfacial tension of such water films. The curves obtained made it appear that the surface-active agents also have an effect on the structure or arrangement of the protein substance. This, however, may or may not be related to the surface-tension reduction of the water.

Nature of Surface-Active Agents

" Surface-active agents are polar compounds and their activity is dependent on the ability of their molecules to become oriented and adsorbed at an interface," according to a definition by Bartell (1939). Ordinarily, molecules of surface-active agents are composed of lyophobic and lyophilic groups. One part of the molecule may consist of a straight or branched hydrocarbon chain or chains which are more or less lyophobic. To this part is usually attached solubilizing groups such as $-SO_4H$, $-SO_3H$, $-PO_4H_2$, $-COOH$, $-OH$ or their corresponding salts which are more or less lyophilic. Since water was the solvent used in this study, *hydrophilic* is the specific term. Because of this dual nature of the molecules they may become oriented at an interface.

The molecules of surface-active agents will have a tendency to locate themselves on the surface of the water in such positions that their hydrophilic groups are directed into the water while their hydrophobic groups extend above the surface. In this position they serve to tie both phases together and thus tend to reduce the abruptness of the phase transition. Whether the reduction of surface tension of this free water in the dough would be the same as when the surface-active agents are in a water solution can of course not be predicted.

General Procedure

Some of these agents were on hand and others were obtained from the manufacturers. Acknowledgment is hereby made for their generous

donations. Only those agents which were readily soluble in water were suited for this work and solutions from 1% to 10% were found to be convenient. From the many agents known to exist, about 35 were selected, and two flours, Tenmarq and Chiefkan, each having medium protein percentages, were used in the preliminary trials. The general procedure was to make trial curves with 100 mg of the agent dissolved in the amount of water used for mixing the dough from 35 g of flour. From the appearance of this trial curve it was possible to decide on the varying amounts of the material that should be used for groups of three or four curves. The smallest amounts used were such as to show a distinct effect on curve characteristics when compared with the curve made from flour and water. The largest amounts were such as to show very marked effects and still retain the main pattern obtained from sound flour.

The Three Main Measures of Curve Characteristics

The three most significant measures of the main characteristics of a curve are: minutes for the recording pen to reach the top of the curve; height of this top, measured in units on the chart; and angle between the rise and fall of the curve. Reference to Figure 1, which shows one curve from Turkey and one from Chiefkan together with the skeleton outline of these curves, will help in following this description. The distance between the curved lines on the chart paper and the rate of move-

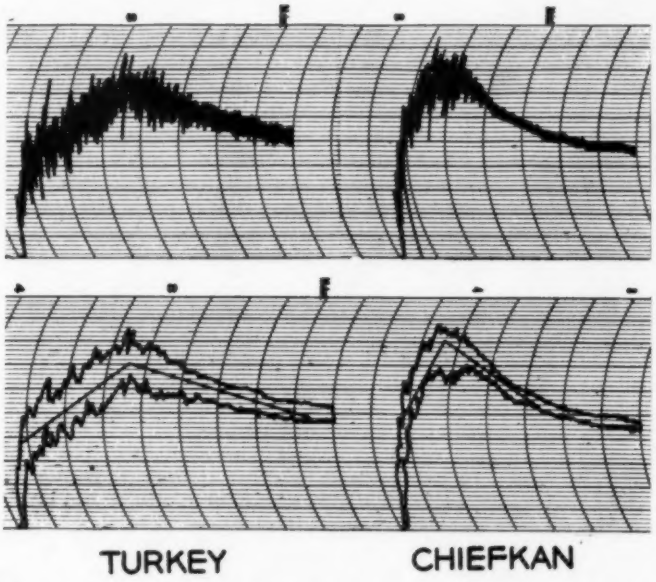

Fig. 1. Curves of Turkey and Chiefkan and their skeletons, illustrating method of describing curve characteristics.

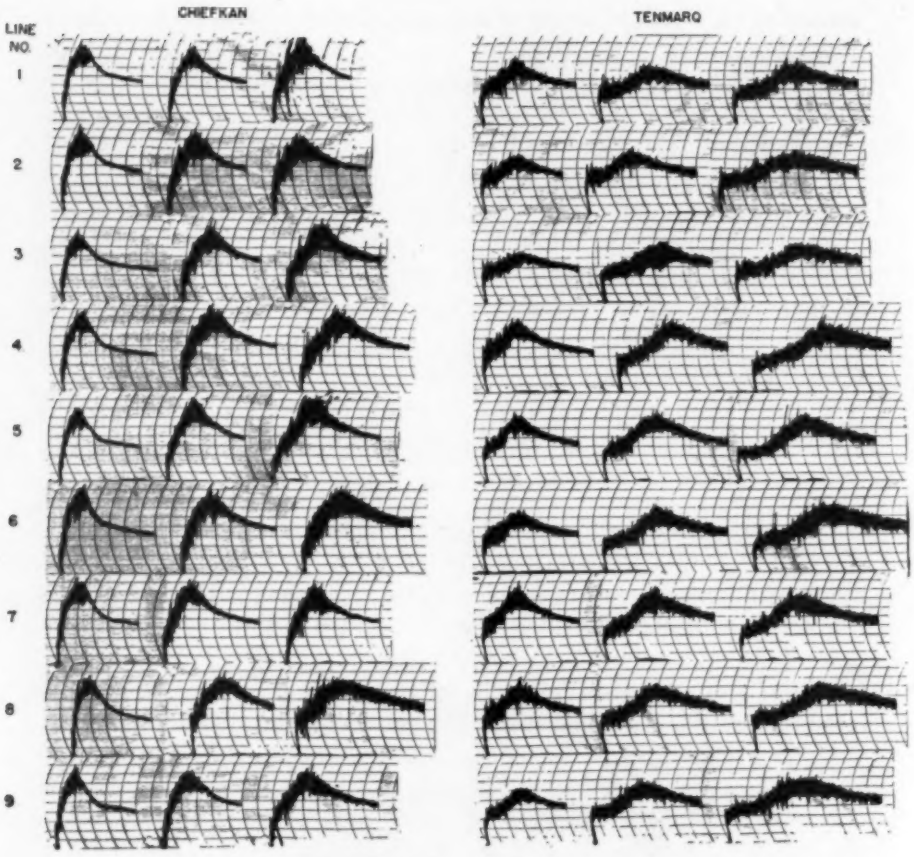

Fig. 2. Effects of surface-active agents.

Agent	Line No.	Chiefkan—agent added			Tenmarq—agent added		
		mg	*mg*	*mg*	*mg*	*mg*	*mg*
Aerosol AY	1	0	40	70	0	40	70
Aerosol OT	2	0	70	100	0	20	60
Dupenol PC	3	0	70	100	0	70	100
Gardinal WA	4	0	100	170	0	100	200
Gardinal WA Flakes	5	0	100	200	0	100	200
Santomerse S	6	0	100	200	0	100	200
Penetrant W 846	7	0	70	100	0	70	100
Tergitol Pen. 4	8	0	100	200	0	60	150
Tergitol Pen. 7	9	0	70	140	0	70	140

ment are such that the time required for the pen to go from one curved line to the other is so near one minute that for practical purposes this is the time unit. The bold horizontal lines divide the chart into ten units and the light lines subdivide these units into five each. Hence, the height of the curve can be measured in units from 1 to 10 and in decimals of these units. The angles made by the up-and-down slopes are

measured by drawing a line in the center of the upslope of the curve and also in the center of the first part of the downslope. The angle which these two lines make near the center of the top of the curve is measured by a protractor and the angle is stated in degrees. This is essentially the method used by Shellenberger (1940).

There are other characteristics in the curves which are significant, such as the general width, the outline of the top, such as rounded or sharp, the curvature of the downslope, and the rate of narrowing of the last portion of the curve (Swanson, 1937). These are used in the visual judging of the curves, but in this discussion the three points mentioned,

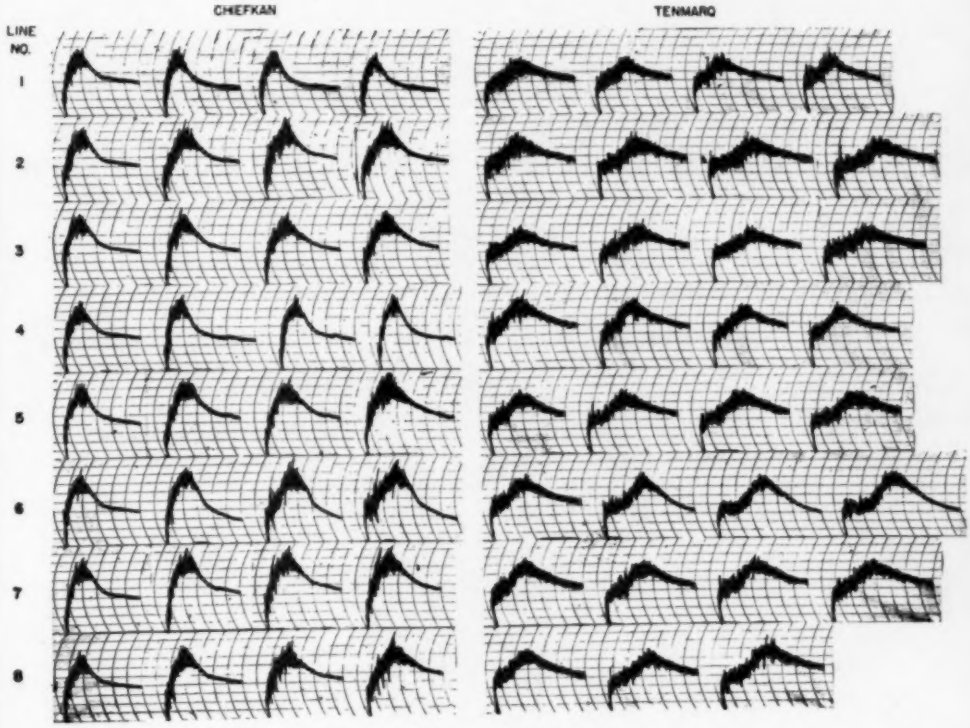

Fig. 3. Effects of surface-active agents.

Agent	Line No.	Chiefkan—agent added				Tenmarq—agent added			
		mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
Aerosol DGA	1	0	70	100	140	0	70	100	140
Aerosol NAD	2	0	70	100	140	0	70	100	140
Dupenol OS	3	0	100	200	300	0	100	200	300
Loupene	4	0	100	140	200	0	100	140	200
Tetra K Phos.	5	0	40	70	100	0	40	70	100
Triton S-18	6	0	100	20	300	0	100	200	300
Wetsit single	7	0	40	70	100	0	40	70	100
X Dry size	8	0	100	200	300	0	100	200	

time of development, height, and angle made by the slopes, will be used in describing modifications caused by the presence of the surface-active agents in the dough.

Effects of the Surface-Active Agents

A study of the curves obtained with the use of about 35 surface-active agents showed that they could be divided into two general classes: one in which the effects were very pronounced and the other in which the effects were not so pronounced. Typical curves in which the effects of the agents were very pronounced are shown in Figure 2 and the legend gives the names and the amounts used for each curve. The small variations in the control curves made with flour and water shown in the first column for each variety are due to the fact that these curves were made on different days with only such atmospheric control as is found in the average laboratory.

TABLE I

SURFACE TENSIONS OF SOLUTIONS OF SOME SURFACE-ACTIVE AGENTS

For curves in Figure 2			For curves in Figure 3		
Agent	Amt. per 21 ml H_2O	Surface tension	Agent	Amt. per 21 ml H_2O	Surface tension
	mg	*dynes/cm*		*mg*	*dynes/cm*
Aerosol AY	70	28.3	Aerosol DGA	140	27.5
Aerosol OT	60	29.9	Aerosol NAD	100	33.1
Dupenol PC	100	37.7	Dupenol OS	300	31.1
Gardinal WA	170	30.6	Loupene	100	30.7
Gardinal EA Flakes	200	30.6	Tetra K Phos.	100	67.1
Santomerse S	150	36.1	Triton S 18	300	31.0
Penetrant W 846	100	34.8	Wetsit single	100	33.4
Tergitol Pen. 4	100	37.6	X Dry Size	300	36.9
Tergitol Pen. 7	140	28.7			

The curves in Figure 2 show that the general effects of the surface-active agents are longer time for development with consequent less-steep upslope, general wider width of the curves, less steep downslope, and a decreased tendency of the curves to become narrow in the last portions. This last effect is particularly true of the curves from Chiefkan, but not so pronounced on Tenmarq. On Chiefkan the influence is so great that the curve characteristics associated with this variety are obliterated. Thus the third column of curves under Chiefkan have a pattern very much like that obtained from Turkey or Tenmarq.

The curves in Figure 3 show that the effects of the surface-active agents used for these curves were much less than those used for the

curves in Figure 2. The patterns of the curves for both Chiefkan and Tenmarq were not greatly altered, although the amounts used for the curves in the fourth column were in several cases larger than those used for the curves in Figure 2.

Surface Tensions of Solutions of Surface-Active Agents

Surface-tension measurements were made with the du Noüy ring method in order to determine whether the differences in the curves of Figure 2 and Figure 3 were due to variations in surface tension. Representative figures given in Table I show that the surface tension of the solutions of these agents were, in general at least, half that of ordinary distilled water, which was 71.9. The agents used for the curves in Figure 2 had about the same effect on surface tension as those used for Figure 3. These figures show that reductions in surface tension were not of first importance. If they had been, the curves in Figure 2 would have been more similar to those in Figure 3.

TABLE II

EFFECT OF INCREASING CONCENTRATION OF AEROSOL OT ON SURFACE TENSION

Milligrams per 21 ml	Surface tensions
	dynes/cm
0	71.9
10	35.8
20	30.8
30	29.9
40	29.5
50	29.5
60	29.5

Furthermore, increasing the amounts of the surface-active agents had a cumulative effect as shown by the curves in Figure 2. As is well known, surface-active agents will decrease the surface tension of water a definite amount, but when this reduction has reached a certain point there will be no further effect. As shown by the figures in Table II, the presence of 10 mg of Aerosol OT in 21 ml of water reduced the surface tension about one-half. Doubling this amount made a further reduction only about one-seventh as great as the first 10 mg. Adding 30 mg or more produced little or no further reduction in surface tensions. However, as shown in Figure 2, and as will be shown later, each additional amount of surface-active agent produced marked effects on the curves.

Effect of the Surface-Active Agent Aerosol OT on Curves of Flours of Varying Protein Content

In the further trials with the effects of surface-active agents, the work was limited to the use of Aerosol OT. While this did not have quite as great an effect as some of the other agents, nevertheless, as seen from the second row of curves in Figure 2, the modifications due to this agent were notable. A particular advantage in the use of this agent is that it is patented and hence its formula is known.

The flours used in these trials were available samples of varying protein content from each of the varieties: Tenmarq, Cheyenne, Chiefkan, and Clarkan. The protein percentages of these samples are given in Table III. The flours were milled from blends composed of a number

TABLE III

PROTEIN IN FLOURS FROM FOUR VARIETIES

Variety	Protein content					
	%	%	%	%	%	%
Tenmarq	15.3	13.3	11.8	10.8	9.3	7.9
Cheyenne	—	13.2	11.2	10.4	9.2	—
Chiefkan	14.6	13.1	—	10.8	9.3	8.7
Clarkan	—	12.8	11.3	9.9	8.7	8.3

of wheat samples of varying protein contents. The wheats were grown in different parts of Kansas.

One problem with flours of varying protein content is to obtain a satisfactory range in water absorption for the curves. It was desired that the upper parts of the curves from the highest-protein samples should be near the top of the chart. The amount of water needed for this height was determined by trials. It was found that as the protein content became lower the most desirable curves from flour and water were obtained by decreasing the absorption 1.5% for each 1% decrease in protein. This held for the higher-protein samples, but as the protein content decreased the effect on absorption became less. The same absorptions as were used for the flour-and-water curves were also used when the surface-active agent was present in the water.

The curves obtained on the flours of varying protein content from the four varieties are given in Figure 4. The first row of curves for each variety was made with flour and water and the second with flour, water, and 50 mg of the surface-active agent, Aerosol OT. The measurements, minutes to reach the top, height in units on the chart, and angles of the up-and-down slopes are given in Table IV.

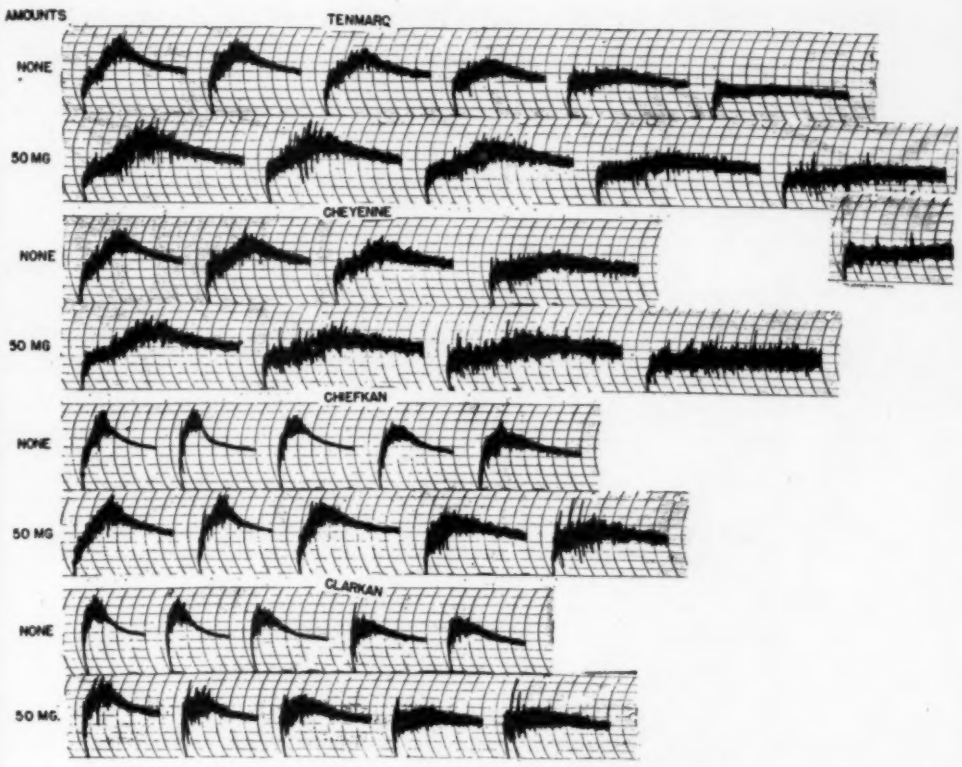

Fig. 4. Effect of surface-active agent on curves of flours of decreasing protein percentages.

First line of curves under each variety: flour + water.
Second line of curves under each variety: flour + water + 50 mg Aerosol OT.

			TENMARQ			
Protein, %	15.3	13.3	11.8	10.8	9.3	7.9
Absorption, %	65.0	62.0	59.8	58.3	56.8	56.0
			CHEYENNE			
Protein, %	13.2	11.2	10.4	9.2		
Absorption, %	61.0	58.0	57.0	57.0		
			CHIEFKAN			
Protein, %	14.6	13.1	10.8	9.3	8.7	
Absorption, %	65.0	63.0	59.3	57.8	57.5	
			CLARKAN			
Protein, %	12.8	11.3	9.9	8.7	8.3	
Absorption, %	58.0	55.8	54.3	53.8	53.5	

The heights of the flour-and-water curves shown in Figure 4 become progressively less as the protein content of the flour decreases. The time required to reach the top was shortest for Clarkan, somewhat longer for Chiefkan, longest for Cheyenne, with Tenmarq somewhat shorter than for Cheyenne. The angles between the slopes increased with decreasing protein content. The time to reach the top became longer as the protein decreased.

TABLE IV

EFFECT OF SURFACE-ACTIVE AGENT ON CHARACTERISTICS OF FLOUR OF DECREASING PROTEIN CONTENT

Curves in Figure 4.

Protein	Absorption	Rate of development		Height to top		Angles of up-and-down slope	
		Flour & water	Aerosol present	Flour & water	Aerosol present	Flour & water	Aerosol present
%	%	*min*	*min*	*units*	*units*	*deg*	*deg*
			TENMARQ				
15.3	65.0	3.0	5.2	8.4	8.0	104	135
13.3	62.0	2.6	4.1	7.8	7.4	113	140
11.8	59.8	3.2	5.7	7.4	6.0	123	150
10.8	58.3	3.0	6.0	6.2	5.6	136	163
9.3	56.8	4.2	8.2	5.4	5.0	158	171
7.9	56.0	4.7	8.0	3.8	4.0	173	175
			CHEYENNE				
13.2	61.0	3.5	5.6	7.8	7.6	115	135
11.2	58.0	3.6	6.1	7.4	6.6	130	155
10.4	57.0	3.9	7.4	7.2	6.2	139	165
9.2	55.0	5.1	8.1	5.8	5.2	162	172
			CHIEFKAN				
14.6	65.0	2.0	3.3	8.4	8.2	87	109
13.1	63.0	1.5	2.1	8.6	8.6	74	94
10.8	59.3	1.6	2.5	8.2	8.0	102	111
9.3	57.8	1.9	3.0	7.2	6.6	105	138
8.7	57.5	2.2	4.3	6.6	5.8	135	155
			CLARKAN				
12.8	58.0	1.2	2.0	8.4	8.0	82	114
11.3	55.8	1.1	2.3	7.6	6.8	102	128
9.9	54.3	1.4	2.2	7.0	6.8	113	129
8.7	53.8	1.3	3.5	5.8	5.4	145	159
8.3	53.5	1.9	3.9	6.0	5.2	145	164

The effect of the presence of the surface-active agent is shown in the second row of curves for each variety in Figure 4. The heights of the curves are slightly decreased, the time required to reach the top of the curves and the angles made by the slopes very much increased, and the general width of the curves also notably increased. The presence of the surface-active agent made the Chiefkan curves approach the characteristics of the flour-and-water curves from Tenmarq.

Effects of Increasing Amounts of Surface-Active Agent on Samples of Decreasing Protein Content

The samples shown in Figure 4 were used to test the effects of using 0, 20, 40, and 60 mg of the surface-active agent Aerosol OT, respectively, for each curve. The curves obtained from Tenmarq and Chey-

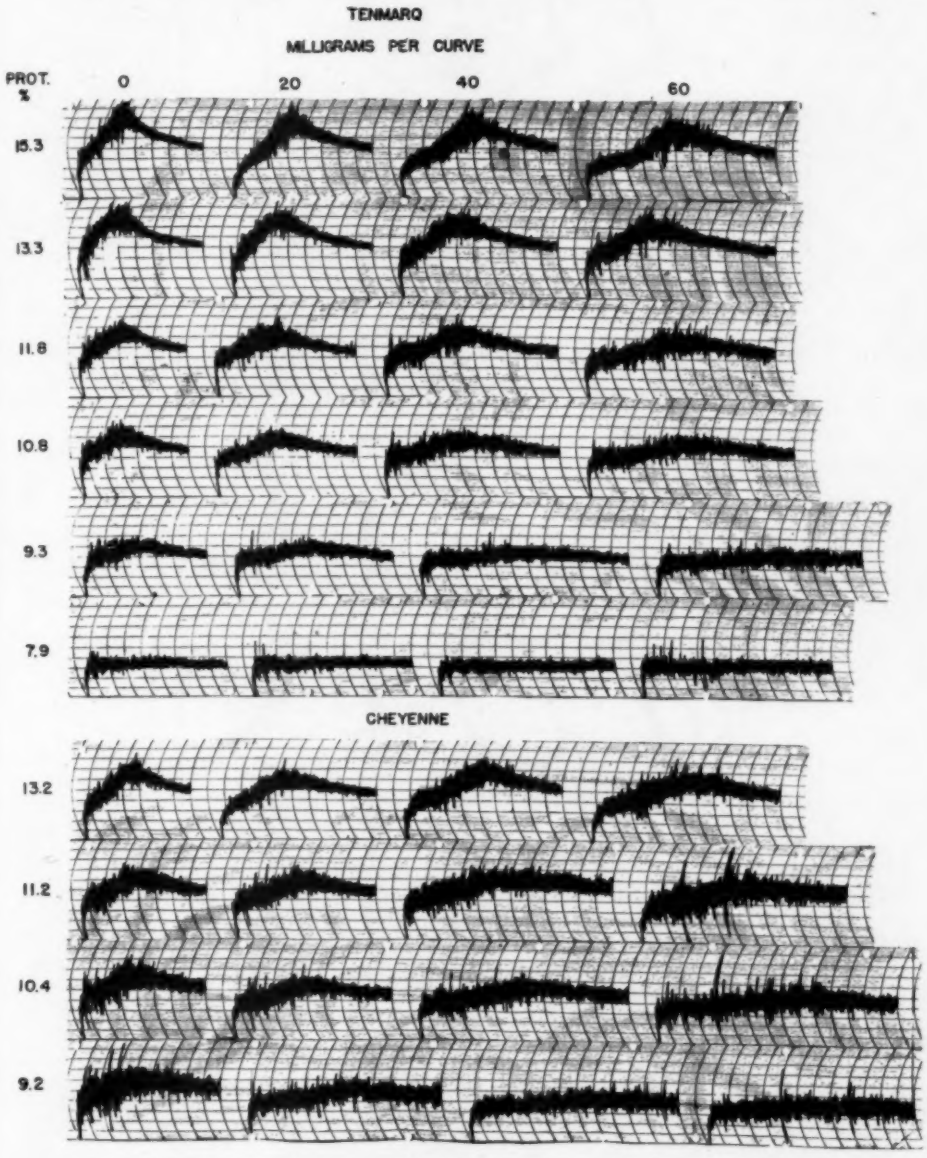

Fig. 5. Effects of increasing amounts of surface-active agent on samples of decreasing protein content—Tenmarq and Cheyenne.

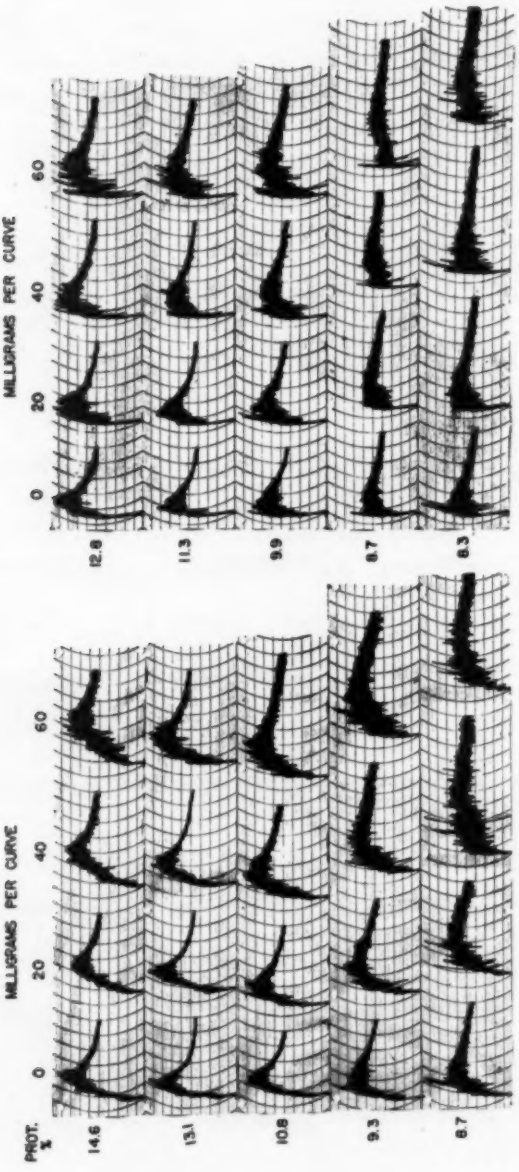

Fig. 6. Effects of increasing amounts of surface-active agent on samples of decreasing protein content—Chiefkan and Clarkan.

TABLE V

Effects of Increasing Amounts of Surface-Active Agent on Samples of Decreasing Protein Content

Curves for Tenmarq and Cheyenne are shown in Figure 5, and curves for Chiefkan and Clarkan in Figure 6.

Protein	Amt. of agent	Rate of development	Height	Angle
%	*mg*	*min*	*units*	*deg*
		TENMARQ		
15.3	0	3.0	9.0	105
	20	4.0	8.2	114
	40	4.5	8.0	127
	60	6.0	7.4	141
13.3	0	2.6	8.8	108
	20	3.3	8.2	119
	40	3.9	8.0	132
	60	4.3	7.8	138
11.8	0	3.0	6.8	125
	20	4.0	6.8	138
	40	5.1	7.0	145
	60	6.2	6.4	157
10.8	0	3.0	6.6	135
	20	4.1	6.4	146
	40	5.2	6.0	158
	60	7.0	5.8	162
9.3	0	3.9	5.4	158
	20	5.5	5.4	163
	40	7.2	5.0	172
	60	8.0	4.8	176
		CHEYENNE		
13.2	0	3.5	7.6	118
	20	4.5	6.8	140
	40	5.2	7.4	140
	60	6.1	6.8	153
11.2	0	3.9	6.8	142
	20	4.7	6.4	153
	40	6.5	6.8	163
	60	7.0	6.4	165
10.4	0	3.4	7.2	138
	20	5.0	6.2	155
	40	6.8	5.8	166
	60	9.0	5.6	169
9.2	0	4.6	6.6	158
	20	7.0	5.4	167
	40	7.7	4.8	172
	60	10.4	4.7	174

TABLE V—*Continued*

Protein	Amt. of agent	Rate of development	Height	Angle
%	*mg*	*min*	*units*	*deg*
		CHIEFKAN		
14.6	0	2.0	8.4	87
	20	2.5	7.8	107
	40	3.0	8.0	107
	60	3.5	7.8	120
13.1	0	1.3	8.4	75
	20	1.7	8.4	82
	40	1.8	8.4	95
	60	2.2	8.8	89
10.8	0	1.6	8.4	86
	20	1.9	8.4	107
	40	2.0	8.6	97
	60	2.7	8.2	120
9.3	0	1.5	7.8	115
	20	2.3	7.8	117
	40	3.0	7.4	129
	60	3.4	7.6	136
8.7	0	1.7	6.8	126
	20	2.7	6.8	131
	40	4.0	6.4	157
	60	4.0	6.2	152
		CLARKAN		
12.8	0	1.3	9.0	89
	20	1.6	8.2	102
	40	1.7	8.6	105
	60	2.3	8.4	118
11.3	0	1.2	7.4	97
	20	1.5	7.4	105
	40	1.9	7.0	121
	60	2.4	7.0	132
9.9	0	1.1	7.2	110
	20	1.6	7.2	109
	40	2.3	6.8	136
	60	2.4	7.0	134
8.7	0	1.9	5.8	149
	20	2.6	5.8	156
	40	3.0	5.6	160
	60	3.9	5.4	160
8.3	0	2.0	6.0	151
	20	2.3	6.0	151
	40	2.2	6.0	161
	60	3.2	6.0	160

enne are given in Figure 5 and the curves from Chiefkan and Clarkan are given in Figure 6. The measurements of these curves are given in Table V.

The curves for each variety are arranged in the plates so that the flour-and-water curves are first in each column, and the others follow in order of increasing amount of surface-active agent. The curves for the various rows of each variety are arranged in order of decreasing protein content. Thus the curves in the first row are from the highest-protein flour and the last row of curves from the lowest-protein flour.

Increasing the amounts of the surface-active agent lengthens the time to reach the top, enlarges notably the angles of the up-and-down slopes, and decreases the heights of the curves of Tenmarq and Cheyenne more than of Chiefkan and Clarkan.

Discussion

The surface-tension values obtained (Tables I and II) were in most instances less than half that of water. The viscosity was determined on the same solutions that were used for the surface-tension measurements given in Table I, using an Ostwald viscosimeter. All these solutions had a viscosity very little different from water alone. Hence, as accords with general knowledge, the surface tension and the viscosity of these solutions are independent of each other. A change in viscosity should be evident from the height of the curves obtained when the surface-active agents were present. Actually the heights were changed very little in comparison with the rate of development. Accordingly there must be at least another type of effect operative in the behavior of dough due to the presence of the added substance. Such might be found in the effect of increased wetting of the gluten and starch particles. Lowering of the interfacial tension and increased spreading ability would result from an increase in the adhesional tendency of water toward the gluten particles covered with the adsorbed wetting agent. In this way each gluten particle would become more completely covered with water and perhaps the molecular interstices would be penetrated to a greater extent. This might be a consequence of the interfacial tension reduction. Additional experiments are being conducted to clarify this problem.

The curves presented show that the mixing time was increased by the presence of the surface-active agent. This could be due to its specific action on the protein molecules themselves in effecting varying degrees of intramolecular changes. Such changes could be related to some form of denaturation.

Protein structure and denaturation: According to writers on proteins (Wrinch, 1937; Lloyd and Shore, 1938; Schmidt, 1938) such substances may assume various physical forms, such as globular or polyhedral, laminar and fibrillar. The models of protein molecules, according to Wrinch (1937), allow for the existence of the fibrillar or one-dimensional, the laminary or two-dimensional, and the globular or three-dimensional form. The three-dimensioned molecules are built up from laminary or two-dimensional cyclised polypeptides which in turn have been formed from fibrillar or one-dimensional molecules. The flat cyclised polypeptides may be held together in piles of two or more deep alternately by direct backbone and side chain linkages. The three-dimensional protein molecules are built by bending round the cyclol fabric so as to make a continuous space-inclosing network in the form of tetrahedrons. The fibrillar forms can pack together to make larger fibrils. In protein films the laminary form is suggested. The globular form exists usually in colloidal solution. Its occurrence in dry flour is possible and its existence in dough is assumed.

Probably a principal effect of the surface-active agent is to further promote the penetration of water into these polyhedral forms and so facilitate their change to other forms. Such changes are implied in denaturation which seems to involve structural alterations of the protein molecules. This can be done according to Lloyd and Shore (1938) by various agents including mechanical agitation, and the action of surface-active agents. Denaturation appears to be accompanied by change in molecular form such as from the globular to the fibrous. This may account for the changes in viscosity which accompany denaturation.

If some sort of globular forms are assumed, such as those suggested by Wrinch (1937), the wetting agent then acts upon this cagelike structure and ruptures it at its weakest links. Such rupture could take place at the hydrogen bonds, the -S-S linkages, and possibly even at the peptide linkages. This rupture would cause a straightening out of the structure into long fibrous patterns. These become more and more fibrous as a result of action of the surface-active agent supplemented by the continued mechanical agitation such as takes place in the bowl of the recording dough mixer. In addition to this, the protein molecule itself contains numerous chemically active groups. These may decompose, rearrange, or react with themselves or with the agent. The latter effect could be a rather prolonged one, due to the time required to disrupt the cagelike protein structures. This process in turn would produce new active groups that were previously either chemically bound into the structure or because of spatial effects were unavailable until exposed by denaturation process. The time element involved in these changes may

account for the lengthening of the period of development shown in the curves when a surface-active agent is present.

Adhesive function of water films in dough: Dough is a coherent mass because of the forces residing in the interfacial water films. These films cover the starch granules as well as the protein particles. The gluten fibrils consist of interwoven protein particles or micelles which entrap the water. Mechanical action such as results from the action of the recording mixer will rearrange the micellar structure. The net result is an increase of surface which would decrease the amount of free or increase the amount of bound water.

Effect of decrease in surface tension due to the presence of the surface-active agents: The presence of the surface-active agent in the water films of the dough promotes the increase of protein surface through their denaturing action, resulting in uncoiling the protein micelles. This spreading of the water films over greater increased protein surfaces would decrease the amount of free water and alter the viscosity of the dough. Such alterations in viscosity, which should result in greater curve heights, were not uniformly found when the surface-active agent was present. This may be due to the weakening of the protein or gluten strands as a result of denaturation by the surface-active agent.

Summary

Surface-active or interfacial-tension-reducing agents modify the characteristics of the curves made by the recording dough mixer to such an extent that the patterns associated with such varieties as Chiefkan may be made similar to the patterns of Turkey or Tenmarq. The most notable effects are to increase the time of devolpment, increase the angles of the up-and-down slopes, and decrease the rate of breakdown of the dough. The smooth narrow characteristic of the last part of Chiefkan curves was completely obliterated.

The surface-active agents reduced the surface tension of water about one-half or more. The effects on the doughs as shown in the curves cannot be explained solely by decreases in the surface tension. The effects of the agents were greater as the concentration of the agent increased. The surface-tension reduction of water proceeds to a certain point after which further additions of the agent produce no further decrease. Some agents had very little effect on the curve patterns, yet they reduced the surface tension of water to about the same extent as those which had marked effects.

The effects of these agents on the curve patterns may be due to their ability to alter the configuration or form of the protein molecule. This change in the protein molecule may be a consequence of interfacial ten-

sion reduction by allowing greater intramolecular penetration of the water. This, together with the mechanical pull-fold-repull action on the dough, may break various bonds, changing the polyhedral molecular form through laminar to fibrillar. Those agents which do not have a marked effect on the curve patterns may have a composition which is less effective in causing this denaturation.

The curve patterns from high-protein flours were changed more than the patterns from low-protein flours. This indicates that the agents affect the protein principally and would support the view that the effects are due to some form of protein denaturation.

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THIAMIN RETENTION IN SELF-RISING FLOUR BISCUITS

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Published information on thiamin retention in "baking powder" products is so scarce that systematic investigation is justified. Copping and Roscoe (1937) found no demonstrable amount of vitamin B_1 remaining in bread, as indicated by the rat-growth method, when baking powder was used in place of yeast. Their "baking powder" bread consisted of flour mixed with 1.25% salt, 1.51% calcium acid phosphate, and 1.07% sodium bicarbonate. The dough was baked at once for 45 minutes at 450°–480°F. They cited the fact that Kent-Jones reported a similar dough as having a pH of 6.7–7.2, whereas yeast dough registered 5.5–5.9. Copping and Roscoe attributed the destruction of vitamin B_1 to its exposure to a mildly alkaline medium at a temperature of 100°C for a relatively long time.

It is well known that thiamin shows considerable resistance to heat in an acid or slightly acid medium. Exposure to temperatures of 100°C in these conditions causes little, if any, loss. However, in an alkaline medium there is extensive destruction even at low temperatures. As self-rising flours produce biscuits slightly on the alkaline side of neutrality, and baking time is relatively short, it might be expected that destruction of thiamin would be relatively small. The work reported here is a study of factors which affect the survival of thiamin in self-rising flour biscuits. Experiments were limited to the use of one flour. In order that they might be applicable to any flour, the tests involved the determination of the amount of added synthetic vitamin, thiamin hydrochloride, which is recovered in the baked biscuit irrespective of native thiamin. The thiochrome method of assay for thiamin was used throughout.

Methods

Self-rising flour and biscuits were prepared by the procedures outlined in Cereal Laboratory Methods (1941) for testing self-rising flour except where otherwise noted. The flour was a commercially milled, bleached product of about 60% extraction. To a weighed portion of self-rising flour was added 12% (flour basis) of hydrogenated shortening, which was mixed in by hand. Water or milk was added and the dough was mixed by machine. It was rolled out, cut, and panned as

TABLE I

REPLICATE ASSAY ON SELF-RISING FLOUR BISCUITS IN THE pH RANGE FOUND IN COMMERCIAL SAMPLES

(Milk used as the liquid; each gram of crumb except the blank received 3.9 μg of added thiamin.)

Neutralizing value	Self-rising flour composition			Total B_1 by assay			Total B_1 less B_1 of control bake			Recovery of added thiamin			Average recovery of thiamin	pH of crumb
	Soda	Anhyd. phos.	Salt	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3		
	%	%	%	μg/g	μg/g	μg/g	μg/g	μg/g	μg/g	%	%	%	%	
90	1.545	1.705	2.0	3.46	3.48	3.35	2.99	2.76	2.84	77	71	73	74	7.4
85	1.495	1.755	2.0	3.59	3.75	3.66	3.12	3.13	3.15	80	79	80	80	7.2
80	1.445	1.805	2.0	3.66	4.02	4.11	3.19	3.40	3.60	82	87	92.5	87	7.0
71.5	1.355	1.895	2.0	3.90	4.34	4.09	3.43	3.72	3.58	88	95	92	92	6.8
80	1.445	1.805	2.0	0.47	0.62	0.51	—	—	—	—	—	—	—	7.0

specified except that the dough pieces were placed in contact with each other in a round pan. Baking was done on the rotary shelf of an electric oven at 450°F; water biscuits were baked for 15 minutes, milk biscuits for 12 minutes. Upon removal, the biscuits were cooled, broken into small pieces, air-dried to constant weight, and ground through a food chopper.

Thiamin was determined on the air-dried, ground biscuits by the thiochrome method (Cereal Laboratory Methods, 1941) with modifications as suggested by the Food and Nutrition Committee of the National Research Council (unpublished).

Experimental

It was found early in this work that the same recovery values were obtained when the synthetic vitamin was blended with the flour as when it was dissolved in a portion of the liquid of the dough. As error might occur due to dusting if thiamin were blended into the flour, it was added as a solution. In all tests reported here 2.16 mg of thiamin per pound of flour was added. In each group of bakings, biscuits receiving no thiamin were included and they were assayed for native thiamin, this value being subtracted from that of biscuits receiving thiamin. The percentage recovery of the added thiamin was then calculated by use of records on the weight of ingredients, the weight of cut dough pieces before baking, and the weight of the baked and air-dried biscuits.

In preliminary tests the following variations in baking procedure were found not to affect the recovery of thiamin:

Machine vs. hand mixing of doughs
Single rolling vs. one or two foldings with subsequent rollings
Immediate baking vs. benching of doughs for ½ hour before preparing for the oven
A round pan with doughs touching vs. an aluminum sheet with doughs spaced
Rotary shelf vs. a wire rack for support of pans in oven
Electric oven vs. gas oven at equivalent temperatures and times of baking
Baking time varying from 9 minutes to 17 minutes, water doughs.

The size of the biscuit had some effect on thiamin recovery. Biscuits of normal size made by rolling a dough out between supporting strips ⅜-inch high resulted in 88% recovery, whereas only 79% thiamin was recovered when ¼-inch strips were used to obtain thin, crusty biscuits. The ⅜-inch strips were used in all tests herein reported.

Assay for thiamin was made with a representative sample of the ground, air-dried biscuit in all cases. However, in one test the crust assayed 2.46, whereas the crumb when plucked out assayed 3.10 μg of thiamin per gram of air-dried product. In another case biscuits baked so as to have deeply browned, partially burned crusts showed decreased thiamin recovery. Overbaking was avoided in the reported tests.

The recovery of thiamin in biscuits made from self-rising flours containing varying ratios of soda to phosphate was studied. Table I presents replicate assays on self-rising flour biscuits in which the ratio of soda to anhydrous calcium acid phosphate was the variable. Each assay value represents triplicate determinations. The average of the standard deviations of replicated determinations was 3(%), which indicates average recoveries to be true values. The amount of thiamin recovered was found to be in proportion to the acidity of the biscuits within the range of pH studied. Table II gives additional evidence that the amount of thiamin surviving the baking process is dependent on the pH. In this table, however, the biscuits were made with water as the liquid, so that pH values and thiamin retention in the biscuits made with anhydrous phosphate are slightly lower than those shown in Table I, where milk was the liquid. The two phosphates were commercial products, the anhydrous calcium acid phosphate having a titration of 86.0 and the calcium acid phosphate monohydrate, 83.6. The ratio of soda to phosphate was varied as before, but the total of soda plus phosphate was held constant in order to prevent any appreciable variation in the amounts of total residual salts.

TABLE II

EFFECT OF ACIDITY AND ALKALINITY ON THIAMIN RECOVERY IN SELF-RISING FLOUR BISCUITS MADE WITH WATER

Neutralizing value	Anhydrous calcium acid phosphate + soda = 3% of flour weight		Calcium acid phosphate monohydrate + soda = 3.625% of flour weight	
	Biscuit pH	Thiamin recovery	Biscuit pH	Thiamin recovery
		%		%
100	8.3	49	8.4	28
90	7.7	76	7.7	60
80	7.3	81	7.3	67
70	7.0	85	7.0	82
60	6.7	91	6.7	80

Table II indicates that optimum thiamin recovery is attained in biscuits that are neutral or slightly acid. The data also show greater thiamin retention at the same pH level in the anhydrous phosphate series. It was thought that greater destruction may have occurred in the monohydrate series during the baking of the biscuits. A test was made wherein biscuits were removed from the oven at consecutive time intervals and the pH of the crumb was obtained after cooling. It was found that a maximum in pH values occurring after 4 to 5 minutes of baking is followed by a rapid drop after 7 to 8 minutes to slightly above the final pH. The time at which the maximum pH occurs is of brief

duration and coincides with the attainment of a temperature of 85°–95°C within the biscuit.[1]

It will be observed in Table III, representing water biscuits, that phosphate of the monohydrate type causes a distinctly alkaline condition to exist temporarily during baking, whereas the anhydrous phosphate causes only a slightly alkaline condition.

TABLE III

MAXIMUM pH ATTAINED IN BISCUITS DURING THE BAKING OF SELF-RISING FLOUR DOUGHS

Self-rising flour formula			Liquid used in baking	Maximum pH during baking	pH of fully baked biscuit crumb
Soda	Phosphate	Salt			
%	% (Anhydrous)	%			
1.25	1.5	2.0	Water	7.5	7.3
1.25	1.5	2.0	Milk	7.35	7.2
1.25[1]	1.75 (Monohydrate)	2.0	Milk	7.1	6.9
1.5	1.875	1.75	Water	8.25	7.25
1.5	1.875	1.75	Milk	7.75	7.2
1.5[1]	2.125	1.75	Milk	7.3	7.0

[1] Enriched self-rising flour formulas recommended.

Table IV supplies thiamin recovery values with biscuits baked under like conditions. It may be concluded that reactions of the anhydrous phosphate with soda within the baking biscuit are more favorable to the retention of thiamin. The use of milk in place of water reduced the maximum pH attained with both types of phosphates and materially improved thiamin retention. The most favorable conditions existed in milk biscuits made from self-rising flour, as proposed for enrichment purposes.

The formula for the commercial manufacture of enriched self-rising flour should be such that an optimum retention of thiamin will be obtained in the baked biscuits. It would not be practical commercially to mix self-rising flours so that a pH of say 6.5 would be obtained in those made with water as this acidity would tend to result in a slightly metallic acid taste and the cost would be excessive. A pH of 7.1 in biscuits made with water seemed a suitable value. Table IV presents comparative results of bakings made with the present standard formulas and by enriched flour formulas proposed for self-rising flours. Both water and milk biscuits were baked in duplicate twice; the average recovery values represent four separate bakings with triplicate assay on

[1] The temperature attained during the baking of biscuits was previously reported from these laboratories (1938, Cereal Chem. 15: 843–45). As bakings were conducted under like conditions of technique, oven temperature, and type of oven, the data there reported may be applied here.

TABLE IV

THIAMIN RECOVERY IN BISCUITS MADE FROM THE PROPOSED ENRICHED SELF-RISING FLOUR FORMULAS AND THE PRESENT STANDARD COMMERCIAL FORMULA

(Each test represents triplicate assays on duplicate bakings)

Formula	Soda	Calcium acid phosphate	Salt	Liquid	Test 1		Test 2		Average thiamin recovery
					pH	Recovery	pH	Recovery	
	%	%	%			%		%	%
Standard	1.25	Anhydrous 1.5	2.0	Water	7.4	76	7.3	78	77
Standard	1.25	Anhydrous 1.5	2.0	Milk	7.3	78	7.1	84	81
Enriched	1.25	Anhydrous 1.75	2.0	Water	7.0	87	7.1	77	82
Enriched	1.25	Anhydrous 1.75	2.0	Milk	7.0	86	7.0	83	84.5
Standard	1.5	Monohydrate 1.875	1.75	Water	7.3	69	7.25	73	71
Standard	1.5	Monohydrate 1.875	1.75	Milk	7.1	79	7.1	80	80
Enriched	1.5	Monohydrate 2.125	1.75	Water	7.0	79	7.0	75	77
Enriched	1.5	Monohydrate 2.125	1.75	Milk	6.9	89	6.9	80	84.5

each set. It will be noted that milk biscuits resulted in pH values 0.2 unit lower than water biscuits and retained an average of 6% more thiamin. There was less spread in thiamin recovery between water and milk biscuits when the self-rising flour contained the anhydrous phosphate rather than the monohydrate. The proposed self-rising flour formulas in which 0.25% additional calcium acid phosphate is included with the present standard formulas resulted in thiamin recovery of 85% when biscuits were made with milk as the liquid.

Discussion

The thiamin value of enriched self-rising flour biscuits when baked under conditions favorable to the optimum preservation of thiamin may be approximated. For practical purposes 80% to 85% of the added thiamin may be used for calculating thiamin in biscuits. The loss of 15% to 20% of the 2.16 mg added per pound of flour, or 0.32 mg, represents approximately the amount of native thiamin in flour, so that the original amount of thiamin added is the amount in the final biscuit.

Thiamin recovery may vary somewhat with the type of baked product, but it appears that optimum retention will be attained if the pH does not become greater than 7.1–7.2 during the baking process. It will be of interest to compare the results reported here with those obtained with other self-rising flour products.

It may be predicted from this work that the use of phosphated flour instead of plain flour will improve greatly the retention of thiamin in biscuits made with baking powders as most baking powders result in biscuits considerably above 7.0 pH.

Summary

The recovery of thiamin added to self-rising flour doughs is shown to be in proportion to the acidity of the biscuits; the lower the pH, the better the recovery. About 85% of the thiamin added survives the baking process if the pH of the baked product is 7.1 or lower.

Commercial self-rising flour formulas, if revised so as to include 0.25% additional calcium acid phosphate in the formula for enriched self-rising flour, would result in optimum retention of thiamin in the finished biscuits.

The use in self-rising flour of calcium acid phosphate of the anhydrous type results in slightly less loss of thiamin during the baking process than the use of the monohydrate. Biscuits made with milk show better thiamin retention than those made with water. Baking conditions and methods of dough preparation have no appreciable effect on thiamin recovery provided a thin, crusty biscuit is not made.

Acknowledgment

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A STUDY OF THE NET WEIGHT CHANGES AND MOISTURE CONTENT OF WHEAT FLOUR AT VARIOUS RELATIVE HUMIDITIES [1]

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The significance of the moisture content of wheat and wheat products in relation to keeping quality and to changes in weight during storage has stimulated many studies of the changes in moisture content and net weight of such products under different conditions of storage. The majority of the experiments on flour reported in the literature—Willard (1911); Guthrie and Norris (1912); Sanderson (1914, 1915); Swanson, Willard, and Fitz (1915); Stockham (1917); Herman and Hall (1921); Frank and Campbell (1922); Arpin and Pecaud (1923); Frank (1923); Smith and Mitchell (1925); Fairbrother (1929); Thiessen (1933); Krtinsky (1937); Cathcart and Killen (1939), and others—have dealt with variations in net weight and moisture content when flours were exposed to varying influences of the atmosphere as encountered under natural storage conditions for different periods of time. These studies have clearly established that flour, in common with wheat and other cereal products, is hygroscopic, its moisture content fluctuating with changes in the relative humidity of the surrounding atmosphere. Because of its more finely divided state and the method of handling, flour responds more readily to changes in atmospheric humidity than does bulk grain. Small packages exhibit greater and more rapid changes in net weight and moisture content than larger ones when stored under the same conditions. In regard to the influence of the container, Krtinsky (1937) found a greater rate of moisture loss in jute than in paper bags

[1] Paper No. 1930, Scientific Journal Series, Minnesota Agricultural Experiment Station.

but Cathcart and Killen (1939) in studies on flour stored in paper-lined, jute, grain, and cotton bags found that the kind of sack had little effect on the variation in moisture content.

Frank (1923) found that while there was no appreciable difference in the rate of increase in the weight of moisture-free hard and soft winter wheat flours, hard winter wheat flour of normal moisture content retained more moisture than soft winter wheat flour exposed to a dry atmosphere.

In general, the experiments under uncontrolled storage conditions indicate that flour as normally packed will lose weight unless the relative humidity of the atmosphere is in the region of 60% or more. Moreover, the experiments of Fairbrother (1929) have shown that flour subjected to low relative humidities absorbs moisture slowly on re-exposure to high humidity and does not recover all the moisture lost; for example, a flour sample after re-exposure to an original relative humidity of 75% for 17 days failed to recover the weight lost during one night at 40% relative humidity. Fairbrother pointed out that, even at normal temperatures, the drying of flour apparently permanently reduces its hydration capacity. In this connection, Smith and Mitchell (1925) found that flour samples which were oven-dried after exposure to the laboratory atmosphere for 42 hours failed to regain all the weight lost during the drying period upon re-exposure to the laboratory atmosphere.

A few investigators—Bailey (1920); Fairbrother (1929); and Anderson (1937)—have determined the hygroscopic equilibria of flour in atmospheres of controlled relative humidity. Bailey found that the moisture content of patent flour exposed to atmospheres of 30, 50, 70 and 80% relative humidity at 25°C (77°F) contained approximately 5.2, 8.0, 12.1, and 15.0% moisture respectively at equilibrium, the curve representing this relation being parabolic in shape. These results were obtained by exposing 5-gram flour samples in aluminum moisture dishes over aqueous sulfuric acid solutions of the required concentration to provide the desired humidity levels at 25°C and determining the moisture content of the flour, after equilibrium had been attained, by a vacuum oven procedure.

In Fairbrother's (1929) controlled humidity experiments, conducted by exposing flour of 13.1% moisture content (method not stated) at approximately 25°C to air in contact with definite concentrations of sulfuric acid and calculating the equilibrium moisture from the change in weight, a linear relation was found between hygroscopic moisture and relative humidities varying between 15% and 90%. The equilibrium moistures obtained by Fairbrother were much higher than those secured by Bailey; for relative humidities of 30, 50, 70 and 80% Fairbrother found the

hygroscopic moisture to be approximately 9.4, 10.7, 13.8 and 15.5% respectively. Anderson (1937) determined the equilibrium relative humidity of hard winter wheat and wheat stocks by placing samples of varying moisture content in closed containers and reading the relative humidity of the atmosphere over the samples with a Crova hygrometer after equilibrium had been established. His hygroscopic equilibria values for hard winter wheat flour cover the range between 50% and 75% relative humidity; at 50% and 75% relative humidity the equilibrium moistures were 12.9% and 15.4% respectively, which exceed those reported by Fairbrother.

From this brief survey of the literature it is clear that wheat flour is hygroscopic, the rate of change in net weight and moisture content being dependent upon the size of the package and the difference between the moisture content of the flour and the equilibrium moisture corresponding to the existing relative humidity under the storage conditions. There is, however, a distinct lack of agreement regarding the effect of different kinds of containers on the rate at which equilibrium is attained and, of even more importance, in the equilibrium values themselves. The discrepancy between the data reported by Bailey (1920), Fairbrother (1929), and Anderson (1937) may be due to differences in the hydration capacity of the flours used or in the technique employed in determining relative humidity and moisture content. Much of the data on changes in the moisture content of flour under natural storage conditions was obtained prior to the official adoption and widespread use of the vacuum oven and 130°C one-hour air-oven methods for determining the moisture content of cereal products.

These considerations prompted the present investigation, the object of which was to determine as carefully as possible the changes in net weight and moisture content of wheat flour packed in different sizes of paper and cotton sacks and stored under controlled humidity conditions until equilibrium was attained. The large-scale storage tests were supplemented by laboratory studies designed to determine the hygroscopic equilibria of wheat flour over a wider range of humidities than was practical in the large-scale tests and to ascertain the effects of temperature and previous history of the flour in regard to its moisture content on the hygroscopic equilibria values.

Experimental

In order that the results might be of the greatest practical significance an 83% patent flour, commercially milled from a blend of hard red spring and hard winter wheats for the family trade and normally sold in 5, 10, and 24½ pound paper and cotton sacks, was used throughout

the studies. The protein content of the flour was 10.8% and the ash content 0.40%, both expressed on a 13.5% moisture basis.

Large-scale storage tests: For the large-scale storage tests, two similar specially designed heavily insulated cabinets, each with a storage capacity of approximately 47.5 cubic feet and completely air-conditioned for temperature and relative humidity, were made available to us. The wet- and dry-bulb elements of an air-operated recording regulator—the air-output from which operated pneumatic-electric switches which actuated the various pieces of equipment necessary to condition the air to the desired value—were located at the entrance of an external duct which carried the air

TABLE I

DATA SHOWING ACCURACY OF TEMPERATURE AND HUMIDITY CONTROL IN AIR-CONDITIONED STORAGE CABINETS

	Approximate conditions maintained in the cabinet			
	70°F 35% RH	70°F 45% RH	70°F 60% RH	70°F 75% RH
Minimum dry bulb, °F	70	69	70	60
Minimum wet bulb, °F	55	56	61	63
Maximum relative humidity, %	37	43	60	73
Maximum dry bulb, °F	72	71	72	71
Maximum wet bulb, °F	56	58.5	62	64
Minimum relative humidity, %	35	47	58	70
Mean dry bulb, °F	71	70	71	70
Mean relative humidity, %	36	45	59	72

from the cabinet to the conditioner. In order to assure uniform air distribution, the conditioned air was delivered to the cabinet through a distributing device located in the center of the cabinet ceiling. The wet- and dry-bulb thermometers were checked against a thermometer calibrated by the U. S. Bureau of Standards prior to the commencement of the trials.

The accuracy of the humidity control, as computed from a survey of the wet- and dry-bulb temperature records made during the actual storage trials, is indicated by the data recorded in Table I. The accuracy is greater than would at first be expected from the recorded fluctuations of wet- and dry-bulb readings which were fairly large, especially the former. This is due to the fact that, from observation, the minima and maxima of the wet- and dry-bulb readings occurred simultaneously; this is reasonable since an increase or decrease in the dry-bulb temperature also raises or lowers respectively the wet-bulb temperature. As a conservative general statement it may be said that the dry-bulb temperature was

held to within approximately ± 1°F and the relative humidity to within ± 2% to 3%.

It was originally planned to carry out storage trials at a temperature of 70°F (21.1°C) and relative humidities of 30, 45, 60, and 75% respectively; the actual storage conditions, as shown by the mean values recorded in Table I for the lowest and highest humidities, deviated considerably from those originally planned. In the instance of the lowest humidity, the cabinet was actually adjusted to maintain 30% relative humidity and the storage trials were begun; however, because of the limited capacity of the cooling system, it was necessary to readjust the cabinet to a higher relative humidity (36%). As only two cabinets were available the tests at 45% and 72% and those at 36% and 59% relative humidity respectively were made simultaneously.

For the storage tests 5-pound, 10-pound, and 24½-pound paper and cotton sacks were machine packed consecutively from the flour bin feeding the packing machinery. During the packing operation four flour samples were taken at intervals for determination of original moisture content. The cotton sacks were machine sewn, the 10- and 24½-pound paper sacks machine tied, and the 5-pound paper sacks glue-sealed.

For the trials at 45% and 72% relative humidity 16 paper and cotton sacks of each weight (5- and 10-pound) were packed, while 20 of each kind and size were employed for each of the remaining two humidity levels; half of the above samples were used for storage at each relative humidity. Owing to limitations in cabinet space, only two 24½-pound sacks (one paper and one cotton) were stored under each of the four storage conditions. The sacks were numbered consecutively and weighed to the nearest 0.5 gram on a Gurley precision balance (capacity 25 kg on each pan, sensitivity 60 mg at full load). The weights employed were adjusted, and certified by the Minnesota Department of Weights and Measures to fall within the allowable tolerances of the U. S. Bureau of Standards for "Class C Commercial Test Weights" (which are identical with the tolerances established for Class I technical weights in the range of from 1 to 500 grams).

After all the sacks were weighed, an operation which required approximately two hours, the 24½-pound sacks were placed horizontally on the bottom shelf of the storage cabinet. The 5- and 10-pound paper and cotton sacks were placed vertically on the remaining two shelves, their positions being randomized. This procedure not only completely randomized the sacks assigned to the two simultaneous storage conditions and their location within the respective cabinets, but also the order in which the original weights of the sacks assigned to the two cabinets were obtained. The sacks were weighed after three days' storage and

weekly thereafter for the duration of the trials. Each week one 5- and one 10-pound paper sack and one 5- and one 10-pound cotton sack for each storage condition were selected at random and retained for moisture determinations. Immediately after weighing, the contents of the selected sacks were placed in air-tight cans; later, the contents of each can were thoroughly mixed in a small MacLellan batch mixer and four samples removed and placed in air-tight containers. One sample was employed in this laboratory for moisture determinations in triplicate by both the vacuum oven and 130°C one-hour air-oven methods as described in *Cereal Laboratory Methods* (4th ed., 1941) published by the American Association of Cereal Chemists. As a check on our laboratory results, the remaining three samples were distributed to each of three commercial mill laboratories which determined and reported their moisture values in duplicate by the 130°C one-hour air-oven method.

TABLE II

MEAN EMPTY SACK WEIGHTS FOR THE VARIOUS STORAGE CONDITIONS

Kind of sack	Mean empty sack weight			
	36% RH	45% RH	59% RH	72% RH
	g	g	g	g
5-lb paper	20.0	20.5	20.5	21.0
5-lb cotton	17.0	17.0	17.0	17.0
10-lb paper	39.0	39.5	40.0	40.5
10-lb cotton	26.5	26.5	27.0	27.5
24½-lb paper	66.5	67.0	68.0	69.0
24½-lb cotton	50.0	50.0	51.0	52.0

In order to permit the calculation of the net weight of flour in each size and kind of container, twelve 5- and twelve 10-pound empty paper and cotton sacks and five 24½-pound paper and cotton sacks were placed in the storage cabinets maintained at each of the storage conditions and the weights determined after equilibrium was attained. The mean sack weights are recorded in Table II.

Laboratory studies of hygroscopic equilibria at constant temperature: Employing samples of the same flour used in the storage tests, four series of hygroscopic equilibrium determinations were made, each at 10% intervals, over the range from approximately 10% to 80% relative humidity. Three series were conducted at a constant temperature of 25°C (77°F) with the flour adjusted to initial moisture contents of 6.5%, 12.2%, and 14.7% respectively; the remaining series was made at 37°C (98.6°F) with flour at 12.2% moisture. The bulk portion of flour reserved for these studies was not stored in air-tight containers

and was found to contain 12.2% moisture at the time the samples were taken for the laboratory studies. The subsample of 6.5% moisture was secured by exposing a portion of the above sample in the thermostat at 25°C, while the sample at 14.7% moisture content was obtained by exposing another portion over water at a temperature of 2°C. Each of the subsamples was thoroughly mixed in a MacLellan batch mixer and reserved in air-tight containers.

The hygroscopicity was determined by exposing the flour over sulfuric acid solutions in closed vessels placed in an air thermostat equipped with a fan and maintained at the required temperature to within ± 0.1°C. In adjusting the atmospheres to the desired relative humidities, the vapor pressure data of Wilson (1921) for aqueous sulfuric acid solutions were employed.

In order that the flour samples might be weighed at intervals without removing them from the atmospheric conditions to which they were exposed and to lessen the possibility of errors due to creeping of the sulfuric acid solution, a technique similar to that described by Fisher (1927) in his studies of the rate of drying of wheat flour over concentrated sulfuric acid was employed. The humidity chambers consisted of battery jars approximately $5\frac{1}{2} \times 6\frac{3}{8} \times 10\frac{1}{2}$ inches high. The rim of each jar was ground and fitted with a plate-glass cover $5\frac{3}{4} \times 7$ inches through the center of which a ¼-inch hole was drilled. An aluminum pan equipped with a stirrup was suspended from a nichrome wire passing through the hole of the cover plate, the free end of the wire being formed into a loop. Eight battery jars were placed in a row in the thermostat, the wires passing through small holes drilled in the top of the thermostat and the aluminum pan supported by inserting short glass rods through the loops. A balance from which the left pan was removed was mounted on a drawer track on top of the cabinet. Attached to the left stirrup was a fine nichrome wire which extended through a hole in the bottom of the balance and terminated in a small hook. The balance could thus be brought directly over each of the battery jars. In weighing, the hook was fastened to the loop of the wire supporting the aluminum pan and stirrup (upon which the moisture dishes were placed), and the glass rod removed. When weighing was not in progress, the holes in the top of the cabinet were sealed with small corks and those in the cover plates by two microscope slides each notched on the edge so that they would fit closely around the wire. Stop-cock grease was used to seal the glass joints, care being taken that no grease came in contact with the wire supporting the aluminum pan.

In determining the hygroscopic equilibria, one liter of the required sulfuric acid solution was placed in the battery jar, a 5-g sample of the

flour contained in a standard aluminum moisture dish placed on the supporting aluminum pan, and the jar sealed with the cover plate and microscope slides. Weighings were made at daily intervals until constant weight was attained; in the instance of the lowest humidities, this required approximately two weeks. At the end of each trial, the concentration of the sulfuric acid solution was obtained by determining the density with a Westphal chainomatic balance and the relative humidity computed. The final moisture content of the flour was calculated from a knowledge of the initial moisture content and the change in weight; this procedure was found to give values in excellent agreement with the results obtained by actual determinations of the final moisture contents at equilibrium.

Results of storage trials: In the storage trials irregularities occurred in the tests at 36% and 59% relative humidity. The studies conducted at 36% were commenced with the cabinet adjusted to a relative humidity of 30% but after three days' operation it was found that the capacity of the cooling coil was insufficient to maintain this level against the large quantities of water vapor being contributed by the flour. The initial moisture content of the flour was 13.5% (air-oven method) and from available data it appeared that this level was the approximate equilibrium value for 65% relative humidity. Since a storage cabinet operating at this humidity was temporarily available, the flour sacks were removed from the "30% cabinet," weighed, and transferred to the 65% cabinet in the hope that the weight losses occasioned by three days' storage would be regained, in approximately the same time interval. In the meantime the empty cabinet was readjusted to operate at 36% relative humidity.

The sacks were weighed at intervals over a period of 11 days, when it became obvious from the rate of increase in weight of the 5- and 10-pound sacks that they would not recover their original weight unless stored for a prolonged period, if indeed at all. The 24½-pound sacks, however, more than regained their original weight. Accordingly, one each of the 5- and 10-pound paper and cotton sacks was reserved for a moisture test, and the remainder transferred to the previous cabinet now adjusted to 36% relative humidity. The initial moisture contents of the 5- and 10-pound sacks stored at this humidity level were therefore not uniform and were, as will be shown later, considerably lower than those stored at the other humidity levels.

The irregularity at 59% relative humidity occurred between the eighth and ninth week of storage, three days prior to the weighings for the ninth week and continuing two days thereafter. During this five-day period the mechanism controlling the water sprays failed, with the result that the relative humidity dropped to a low of approximately 44%. The

TABLE III

MEAN PERCENTAGE CHANGE IN NET WEIGHT OF FLOUR STORED AT 70°–71°F (21.1°–21.7°C) FOR VARYING TIMES IN 5-, 10-, AND 24½-POUND PAPER AND COTTON SACKS AT DIFFERENT RELATIVE HUMIDITIES

Storage period	Number of sacks[1]	Percentage change in net weight											
		5-lb sacks			10-lb sacks			5- and 10-lb sacks				24½-lb sacks	
		Paper	Cotton	Paper and cotton	Paper	Cotton	Paper and cotton	Paper	Cotton	Paper and cotton	Standard error[2]	Paper	Cotton
days		%	%	%	%	%	%	%	%	%	%	%	%
FLOUR STORED AT 36% RELATIVE HUMIDITY AND 71°F (21.7°C)													
3	9	−1.21	−1.48	−1.34	−1.19	−1.32	−1.25	−1.20	−1.40	−1.30	.082	−1.66	−1.92
7	9	−2.01	−2.40	−2.20	−1.95	−2.12	−2.03	−1.98	−2.25	−2.12	.075	−2.28	−2.66
14	8	−2.69	−2.94	−2.81	−2.63	−2.74	−2.69	−2.66	−2.84	−2.75	.086	−2.90	−3.32
21	7	−3.07	−3.23	−3.15	−3.03	−3.16	−3.10	−3.05	−3.20	−3.12	.077	−3.34	−3.75
28	6	−3.29	−3.35	−3.32	−3.27	−3.34	−3.31	−3.28	−3.35	−3.31	.078	−3.65	−4.01
35	5	−3.37	−3.38	−3.37	−3.39	−3.41	−3.40	−3.38	−3.40	−3.39	.083	−3.85	−4.16
42	4	−3.30	−3.23	−3.26	−3.31	−3.34	−3.32	−3.30	−3.28	−3.29	.096	−3.89	−4.15
49	3	−3.35	−3.32	−3.34	−3.39	−3.41	−3.40	−3.37	−3.36	−3.37	.058	−4.00	−4.22
56	2	−3.26	−3.20	−3.24	−3.34	−3.35	−3.35	−3.30	−3.28	−3.29	.092	−4.02	−4.19
63	1	−3.14	−3.23	−3.18	−3.32	−3.33	−3.32	−3.23	−3.28	−3.26	—	−3.98	−4.13
70	1	−3.25	−3.30	−3.28	−3.32	−3.99	−3.66	−3.28	−3.64	−3.46	—	−3.99	−4.12
FLOUR STORED AT 45% RELATIVE HUMIDITY AND 70°F (21.1°C)													
3	8	−0.82	−0.93	−0.87	−0.70	−0.76	−0.73	−0.76	−0.84	−0.80	.048	−0.51	−0.60
7	8	−1.25	−1.32	−1.28	−1.06	−1.19	−1.13	−1.16	−1.26	−1.21	.055	−0.82	−0.97
14	7	−1.59	−1.63	−1.61	−1.42	−1.52	−1.47	−1.50	−1.58	−1.54	.049	−1.14	−1.30
21	6	−1.75	−1.77	−1.76	−1.61	−1.70	−1.65	−1.68	−1.73	−1.71	.036	−1.36	−1.51
28	5	−1.84	−1.84	−1.84	−1.73	−1.77	−1.75	−1.78	−1.80	−1.79	.034	−1.51	−1.63
35	4	−1.92	−1.94	−1.93	−1.84	−1.93	−1.89	−1.88	−1.94	−1.91	.067	−1.64	−1.76
42	3	−2.04	−2.08	−2.06	−1.99	−2.00	−1.99	−2.02	−2.04	−2.03	.027	−1.82	−1.91
49	2	−2.16	−2.18	−2.17	−2.10	−2.10	−2.10	−2.13	−2.14	−2.14	.019	−1.94	−2.03
56	1	−2.14	−2.20	−2.17	−2.14	−2.08	−2.11	−2.14	−2.14	−2.14	—	−2.06	−2.10
63	1	−1.96	−2.02	−1.99	−1.95	−1.87	−1.91	−1.96	−1.94	−1.95	—	−1.97	−1.98
67	1	−1.94	−2.00	−1.97	−1.91	−1.86	−1.88	−1.92	−1.93	−1.93	—	−1.94	−1.95
FLOUR STORED AT 59% RELATIVE HUMIDITY AND 71°F (21.7°C)													
3	10	0.12	0.27	0.20	0.20	0.23	0.22	0.16	0.25	0.21	.028	0.18	0.21
7	10	0.19	0.33	0.26	0.25	0.31	0.28	0.22	0.32	0.27	.037	0.24	0.28
14	9	0.32	0.46	0.39	0.40	0.44	0.42	0.36	0.45	0.40	.028	—	—
21	8	0.19	0.28	0.23	0.30	0.31	0.30	0.24	0.29	0.27	.023	0.31	0.33
28	7	0.10	0.23	0.17	0.19	0.23	0.21	0.15	0.23	0.19	.028	0.25	0.25
35	6	−0.11	0.00	−0.06	0.03	0.04	0.03	−0.04	0.02	−0.01	.018	0.12	0.12
42	5	−0.26	−0.15	−0.20	−0.12	−0.12	−0.12	−0.19	−0.13	−0.16	.019	0.00	−0.02
49	4	−0.08	+0.06	−0.01	+0.03	+0.07	+0.05	−0.02	+0.06	+0.02	.022	0.08	0.10
56	3	−0.16	−0.08	−0.12	−0.04	−0.02	−0.03	−0.09	−0.05	−0.07	.020	0.02	0.03
63	2	−1.49	−1.74	−1.62	−1.25	−1.48	−1.36	−1.37	−1.61	−1.49	.051	−0.85	−1.01
70	1	−0.79	−0.68	−0.74	−0.62	−0.62	−0.62	−0.70	−0.65	−0.68	—	−0.50	−0.54
77	1	−0.66	−0.57	−0.62	−0.48	−0.47	−0.48	−0.57	−0.52	−0.54	—	−0.41	−0.43
FLOUR STORED AT 72% RELATIVE HUMIDITY AND 70°F (21.1°C)													
3	8	0.68	0.94	0.81	0.67	0.77	0.72	0.68	0.86	0.77	.045	0.45	0.57
7	8	1.08	1.34	1.21	1.04	1.17	1.10	1.06	1.25	1.16	.050	0.75	0.92
14	7	1.32	1.51	1.42	1.26	1.38	1.32	1.29	1.44	1.37	.041	1.00	1.14
21	6	1.46	1.63	1.54	1.40	1.52	1.46	1.43	1.57	1.50	.050	1.16	1.29
28	5	1.45	1.60	1.52	1.41	1.50	1.46	1.43	1.55	1.49	.051	1.22	1.31
35	4	1.43	1.60	1.52	1.44	1.52	1.48	1.43	1.56	1.50	.044	1.28	1.38
42	3	1.49	1.66	1.57	1.51	1.58	1.54	1.50	1.62	1.56	.057	1.32	1.42
49	2	1.64	1.78	1.71	1.69	1.73	1.71	1.66	1.76	1.71	.060	1.45	1.55
56	1	1.61	1.69	1.65	1.74	1.68	1.71	1.68	1.68	1.68	—	1.47	1.57
63	1	1.61	1.64	1.62	1.72	1.67	1.70	1.66	1.66	1.66	—	1.48	1.55
67	1	1.62	1.66	1.64	1.73	1.65	1.69	1.68	1.66	1.66	—	1.50	1.56

[1] The values given in this column represent the number of 5- and 10-lb paper and cotton sacks respectively which were weighed at each storage period. Only one 24½-lb sack of each kind was employed.

[2] The values given in this column represent the pooled standard errors (single determination) for all 5- and 10-lb sacks.

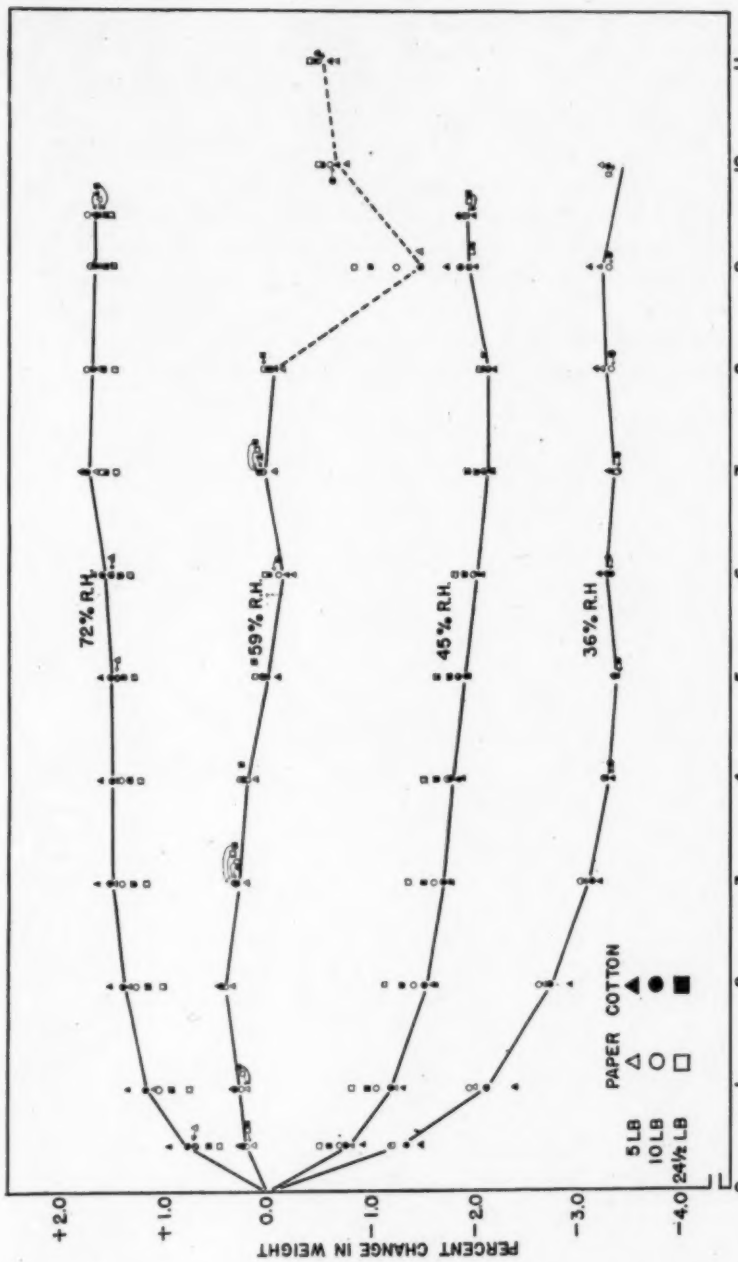

Fig. 1. Mean percentage changes in net weight of flour stored in 5-, 10-, and 24½-pound paper and cotton sacks at 70°–71°F (21.1°–21.7°C) and different relative humidities. (The lines drawn through the scatter of points represent the changes in the mean values for all 5- and 10-pound sacks (paper and cotton) combined. In the storage tests at 59% relative humidity the humidifying apparatus failed three days prior to the weighings for the ninth week and continued for two days thereafter. During this five-day interval the relative humidity fluctuated around 44%.)

humidifying apparatus was repaired during this interval and the storage trials continued for a further two weeks.

The mean percentage changes in net weight of the flour stored in the various sacks for different intervals of time at each of the four humidity levels are recorded in Table III, together with the pooled standard errors (for single determinations) for all 5- and 10-pound sack weight changes at each interval of storage. The percentage changes in weight of the various sizes and kinds of sacks are represented graphically in Figure 1; the lines drawn through the scatter of points represent the mean values for the 5- and 10-pound paper and cotton sacks as recorded in Column 11 of Table III.

The mean results of triplicate determinations of flour moisture content for the various storage conditions are recorded in Tables IV and V.

TABLE IV

MOISTURE CONTENT OF FLOUR STORED AT 70°–71°F (21.1°–21.7°C) IN 5- AND 10-POUND PAPER AND COTTON SACKS FOR VARYING TIMES AT DIFFERENT RELATIVE HUMIDITIES

Storage period	Moisture content [1]								
	5-lb sack			10-lb sack			5- and 10-lb sack		
	Paper	Cotton	Paper and cotton	Paper	Cotton	Paper and cotton	Paper	Cotton	Paper and cotton
days	%	%	%	%	%	%	%	%	%
FLOUR STORED AT 36% RELATIVE HUMIDITY AND 71°F (21.7°C)									
0	12.7	12.6	12.66	12.6	12.8	12.71	12.67	12.70	12.68
7	11.0	10.6	10.78	11.1	11.0	11.07	11.07	10.78	10.92
14	10.5	10.1	10.30	10.6	10.4	10.51	10.57	10.23	10.40
21	10.2	9.9	10.05	10.3	10.0	10.17	10.27	9.95	10.11
28	9.9	9.7	9.82	10.0	9.9	9.94	9.95	9.81	9.88
35	9.8	9.7	9.74	9.8	9.7	9.75	9.81	9.69	9.75
42	9.9	9.9	9.89	10.0	9.9	9.94	9.94	9.89	9.91
49	9.9	9.9	9.86	9.9	9.8	9.89	9.89	9.86	9.87
56	9.9	9.8	9.85	9.9	9.8	9.85	9.90	9.79	9.85
70	9.8	10.0	9.90	9.8	9.8	9.80	9.82	9.88	9.85
FLOUR STORED AT 45% RELATIVE HUMIDITY AND 70°F (21.1°C)									
0	13.3	13.3	13.30	13.3	13.3	13.30	13.30	13.30	13.30
7	12.2	12.0	12.11	12.3	12.1	12.17	12.22	12.05	12.14
14	11.8	11.7	11.78	11.8	11.8	11.83	11.82	11.79	11.80
21	11.7	11.6	11.66	11.8	11.6	11.68	11.75	11.59	11.67
28	11.6	11.5	11.56	11.6	11.5	11.56	11.63	11.49	11.56
35	11.6	11.5	11.53	11.6	11.5	11.58	11.61	11.50	11.55
42	11.4	11.2	11.30	11.3	11.3	11.27	11.31	11.26	11.28
49	11.3	11.3	11.27	11.4	11.3	11.35	11.33	11.29	11.31
67	11.5	11.4	11.43	11.4	11.4	11.42	11.45	11.39	11.42

[1] Air-oven method.

TABLE IV—*Continued*

Storage period	Moisture content [1]								
	5-lb sack			10-lb sack			5- and 10-lb sack		
	Paper	Cotton	Paper and cotton	Paper	Cotton	Paper and cotton	Paper	Cotton	Paper and cotton
days	%	%	%	%	%	%	%	%	%
FLOUR STORED AT 59% RELATIVE HUMIDITY AND 71°F (21.7°C)									
0	13.4	13.4	13.40	13.4	13.4	13.40	13.40	13.40	13.40
7	13.4	13.4	13.41	13.5	13.5	13.48	13.46	13.43	13.44
14	13.5	13.6	13.52	13.5	13.6	13.53	13.48	13.57	13.53
21	13.6	13.6	13.56	13.6	13.6	13.61	13.59	13.58	13.59
28	13.4	13.4	13.43	13.4	13.4	13.44	13.43	13.44	13.43
35	13.4	13.4	13.38	13.3	13.3	13.34	13.36	13.36	13.36
42	13.1	13.2	13.14	13.1	13.1	13.10	13.11	13.12	13.12
49	13.2	13.3	13.24	13.2	13.2	13.24	13.22	13.26	13.24
56	13.2	13.2	13.23	13.2	13.2	13.21	13.22	13.22	13.22
63	12.2	11.8	12.01	12.2	11.9	12.08	12.20	11.89	12.05
77	12.7	12.7	12.70	12.7	12.7	12.70	—	—	—
FLOUR STORED AT 72% RELATIVE HUMIDITY AND 70°F (21.1°C)									
0	13.3	13.3	13.30	13.3	13.3	13.30	13.30	13.30	13.30
7	13.9	14.1	14.01	13.8	13.9	13.86	13.86	14.01	13.93
14	14.1	14.2	14.19	14.1	14.2	14.15	14.12	14.21	14.17
21	14.4	14.4	14.42	14.2	14.3	14.27	14.32	14.37	14.34
28	14.3	14.3	14.31	14.2	14.3	14.24	14.27	14.27	14.27
35	14.3	14.4	14.38	14.4	14.4	14.36	14.35	14.39	14.37
42	14.4	14.5	14.43	14.3	14.2	14.26	14.33	14.36	14.34
49	14.5	14.5	14.50	14.4	14.6	14.48	14.43	14.55	14.49
67	14.4	14.4	14.41	14.4	14.4	14.40	14.41	14.40	14.41

[1] Air-oven method.

TABLE V

INITIAL AND FINAL MOISTURE CONTENT OF FLOUR STORED IN 24½-POUND PAPER AND COTTON SACKS AT 70°–71°F (21.1°–21.7°C) AND DIFFERENT RELATIVE HUMIDITIES

Time of storage	Storage conditions			Flour moisture content [1]			
	Temperature		Relative humidity	Initial		Final	
				Paper	Cotton	Paper	Cotton
days	°F	°C	%	%	%	%	%
70	71	21.7	36	13.7	13.7	9.9	9.7
67	70	21.1	45	13.3	13.3	11.4	11.4
77	71	21.7	59	13.4	13.4	12.8	12.8
67	70	21.1	72	13.3	13.3	14.2	14.2

[1] Air-oven method.

While moistures were determined in this laboratory by both the vacuum-oven and air-oven methods and in three commercial laboratories, the results were in such excellent general agreement that only the mean air-oven values obtained in this laboratory are presented. For all samples, the air-oven values determined by this laboratory averaged 0.058% lower than for the vacuum-oven method; the experimental errors were virtually identical, the standard error of a single determination being 0.042% for the air-oven and 0.041% for the vacuum-oven method. In the instance of 131 samples, for which strictly comparable air-oven techniques were employed in all four laboratories, the mean moisture obtained by us was 12.163%, and the general mean for the three commercial laboratories was 12.115%. In view of this excellent agreement, it is immaterial, in relation to the general conclusions, as to which moisture data are employed. However, because of its rapidity and convenience the air-oven method is in more general use than the vacuum-oven method in mill laboratories and the results by this procedure were arbitrarily selected for presentation.

With reference to the percentage changes in net weight shown in Table III and Figure 1, it must be recalled that the initial moisture content of the flour stored at 36% relative humidity was not uniform because of the differential response of the various kinds of sacks to the preliminary exposures at 30% and 65% relative humidity. As shown in Tables IV and V, the initial moisture content of the 24½-pound sacks stored at 36% relative humidity was 13.7%, whereas the moisture contents of the 5- and 10-pound sacks ranged between 12.6% and 12.8%. For this reason the data for the 24½-pound sacks have been omitted from Figure 1. The mean initial moisture content of the flour as packed for the storage trials at 45% and 72% relative humidity was 13.3%; for the sacks stored at 59% the mean moisture was 13.4%. These means are based on triplicate determinations made on four samples taken at intervals during packing. Variance analyses revealed that the differences between the moisture contents of the four samples taken during each packing, respectively, were not significant. It can therefore be assumed that the moisture contents for all sacks packed at any one time were identical.

These studies of the percentage changes in weight show that flour responds very readily to changes in relative humidity. The rate of change is relatively rapid during the first few days of storage, the actual rate for any one type of container depending upon the magnitude of the difference between the existing relative humidity and the equilibrium humidity corresponding to the moisture content of the flour.

The initial response to any one set of storage conditions depends upon the size and kind of package. Thus, from Table III and Figure 1

it will be noted that the rate of change in weight decreases with increasing package size and is greater for cotton than for paper sacks; as the storage period is prolonged the cumulative percentage changes in weight for the respective containers tend to equalize. This differential initial response due to the size and kind of sack is most pronounced in the storage trials at 36, 45, and 72% relative humidity where the rate of change is relatively rapid. At 59% relative humidity, the mean rate of change is slow since this humidity level is close to the equilibrium humidity corresponding to the initial moisture content of the flour stored in the 5- and 10-pound sacks. That the differences discussed above are statistically significant has been shown by variance analyses of the data for the 5- and 10-pound sacks for the early storage periods at the different humidity levels; for these periods there was sufficient replication to provide a precise test. The significance of the differences in change in weight of the 5- and 10-pound sacks may also be ascertained by computation from the standard errors for single determinations given in Table III.

The rapidity of the change in weight with changing storage conditions and the differential response of the various sizes and kinds of sacks is perhaps most clearly shown by the data obtained after the failure of the humidity control between the eighth and ninth week of storage at 59% relative humidity. This failure occurred three days before the weighings for the ninth week, the relative humidity rapidly dropping to approximately 44% and fluctuating around this level for five days when the repair was completed. At the eighth week the percentage changes in weights for the different sacks were similar, varying only from + 0.02 to — 0.16%. Upon three days' storage at the lower humidity, the 5-pound cotton sack had decreased 1.74% in weight, the 5-pound paper sack 1.49%, the 10-pound cotton 1.48%, the 10-pound paper 1.25%, the 24½-pound cotton 1.01% and the 24½-pound paper 0.85%. The average cumulative percentage weight change for the 5- and 10-pound paper and cotton sacks was 1.49% or a decrease of 1.42% between the eighth and ninth week. Despite the fact that the repair was completed two days after the weighings for the ninth week, and the sacks re-exposed to 59% relative humidity five days (the same period of time for which they were accidentally exposed to the lower humidity) before the weighings for the tenth week, the average cumulative weight change for the 5- and 10-pound paper and cotton sacks was — 0.68%, a regain of only about 55% of the weight lost. After a further week's storage, there was an additional average regain of only 0.14%. The rate of loss in weight was therefore greater than the rate of regain and the data indicate that the original moisture content would not be reached

except after prolonged storage, if indeed at all. It is of interest to note that the average moisture content for the 5- and 10-pound sacks at the ninth week of storage at 59% relative humidity was 12.05% as compared with 13.22% at the eighth week; thus, the drying of the flour to a 12.0% moisture level apparently altered its hygroscopicity more or less permanently.

The irregularity already mentioned in connection with the storage tests at 36% relative humidity confirms these observations. The weight lost by the 5- and 10-pound sacks in four days at 30% relative humidity was not regained in 11 days' storage at 65%. The original moisture content for all the sacks was 13.4%; upon exposure to 30% and then 65% relative humidity the average moisture content for the 5- and 10-pound sacks was 12.7% and that for the 24½-pound sacks 13.7%. The 24½-pound sacks more than regained their original weight. The reason for this discrepancy in behavior is not clear. While the differential response of the sacks to exposure at 30% relative humidity carried the moisture content of the flour stored in the 5- and 10-pound sacks to a moisture content averaging approximately 0.5% lower than was the case for the 24½-pound sacks, this variation does not appear sufficient to account for the difference in behavior unless the hydration capacity of flour is greatly influenced by relatively slight reductions in moisture content.

Examination of the data given in Table III and Figure 1 does not indicate that any well established equilibrium in net weight has been attained under any of the storage conditions. The behavior of the flour stored at 59% and 72% relative humidity is erratic, particularly at the former level. Careful examination of the wet- and dry-bulb temperature records failed to reveal any trend in the temperature or relative humidity with time of storage which would explain the drifts in the percentage weight changes. Since temperature was held to within 1°F and relative humidity to within 2% to 3%, and the work was carefully performed, it must be concluded that flour is extremely sensitive to slight changes in storage conditions and/or that the hydration capacity of flour varies with time and, accordingly, has no precise equilibrium value even under fixed storage conditions.

The moisture data recorded in Table IV for the 5- and 10-pound sacks follow the same general trends as the percentage changes in weight given in Table III. In fact, the moisture content at each storage period may be calculated from a knowledge of the initial moisture content, the initial net weight, and the corresponding weights at each storage period. These computations have been made and the calculated and determined moisture values found to be in good agreement, the maximum discrepancy being 0.3%.

The moisture content of flour packed in 5- and 10-pound sacks changes quite rapidly when stored at relative humidities which differ appreciably from the equilibrium humidity corresponding to the moisture content of the flour, the rate of change decreasing as the moisture content approaches the equilibrium value. For example, during the first week of storage at 36% relative humidity, the moisture content of the flour fell from an initial mean value of 12.68% to 10.92% or a mean loss of 1.76%; in the same interval of time flour containing initially 13.3% moisture lost an average of 1.16% moisture when stored at 45% humidity while it gained 0.63% moisture upon storage at 72% relative humidity. The rate of change in moisture content during the first few

TABLE VI

HYGROSCOPIC EQUILIBRIA OF FLOUR OF VARYING INITIAL MOISTURE CONTENTS AT DIFFERENT RELATIVE HUMIDITIES AND CONSTANT TEMPERATURES [1]

Temperature 25°C						Temperature 37°C	
Initial flour moisture 6.5%		Initial flour moisture 12.2%		Initial flour moisture 14.7%		Initial flour moisture 12.2%	
RH [2]	EM	RH	EM	RH	EM	RH	EM
%	%	%	%	%	%	%	%
10.6	6.1	10.6	5.9	10.9	6.0	12.0	5.5
20.4	7.5	20.6	8.2	20.8	8.1	22.8	7.3
29.6	8.7	29.6	9.5	29.2	9.3	31.0	8.6
39.2	10.1	39.2	11.0	39.1	10.9	40.9	10.1
49.9	11.5	50.0	12.3	49.4	11.9	51.4	11.3
59.2	12.8	59.5	13.2	59.0	13.5	60.5	12.4
68.7	14.1	68.7	14.3	70.4	14.0	69.2	13.6
78.7	15.9	78.8	16.1	78.4	15.8	78.8	15.4

[1] All moisture determinations were carried out by the air-oven method.
[2] RH = relative humidity. EM = equilibrium moisture.

weeks of storage at each of these humidity levels increases with a decrease in the size of the container and is greater for cotton than for paper sacks. In the storage trials at 59% relative humidity, there is no significant differential response due to the size or kind of sack since this humidity level is close to the equilibrium humidity corresponding to the moisture content of the flour, and the rate of change is relatively slow. Statistical analyses of the data for 45%, 59%, and 72% relative humidity confirmed the above observation.[2] For the trials at 45% and 72% humidity, significant interactions were found between storage period and size and kind of sack, and are a reflection of the fact that initially there is a wide differential response of these different sacks to the storage conditions, but as the storage progresses the moisture values tend to

[2] A statistical analysis was not conducted for the 36% humidity level since the moisture contents for the different sacks were not identical at the commencement of the storage period.

approach a common equilibrium independent of the nature or capacity of the sack. Insignificant interactions for size and kind of sack showed that the relative behavior of the flour stored in the 5- and 10-pound sacks is the same whether they are made of paper or cotton.

Results of laboratory determinations of hygroscopic equilibria: The mean results of duplicate determinations of the hygroscopic equilibria of the flour at initial moisture contents of 6.5%, 12.2%, and 14.7%

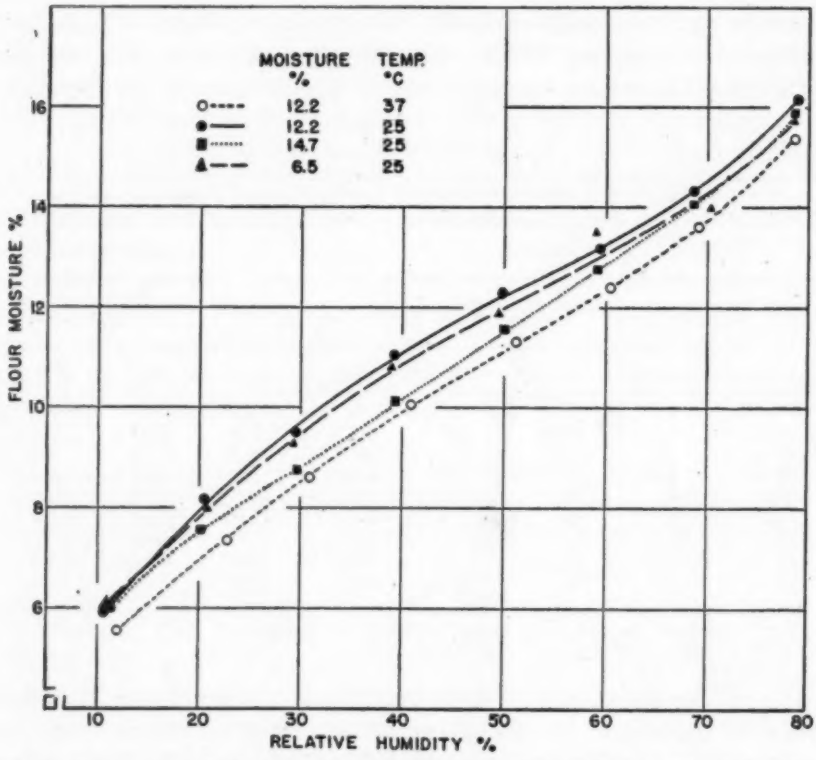

Fig. 2. Hygroscopic equilibria of flour at 25°C (77°F) and 37°C (98.6°F) conditioned to various initial moisture contents.

moisture at various relative humidities are recorded in Table VI and represented graphically in Figure 2. These results show that the hygroscopic equilibrium of a particular flour at any given humidity is not a constant value but depends on its original moisture content and the temperature at which the equilibrium is determined. The lower hygroscopicity of the flour with an initial moisture content of 12.2% determined at 37°C (98.6°F) as compared with 25°C (77°F) would of course be anticipated from the fact that adsorption reactions are characterized by a negative temperature coefficient. The hygroscopicity

values for 25°C (77°F) are not in the order of the original moisture contents of the flour, the sample of 12.2% moisture having the highest and that of 14.7% moisture the lowest hygroscopicity. Whether or not this displacement is due to the conditioning of the high-moisture sample at 2°C (35.6°F) (in order to preclude mold development), whereas the sample at 6.5% was produced by air-drying at room temperature, it is impossible to say but the data indicate that, in common with other biocolloids, relatively minor changes in treatment have an important influence on the hydration capacity of flour. In these experiments, an S-shaped curve best expresses the relationship between relative humidity and hygroscopicity, whereas Bailey (1920) secured curves having the shape of a simple parabola and Fairbrother (1929) found a linear relationship over a similar range in relative humidity. In a theoretical discussion of the water relationships in colloids, Briggs (1931) points out from thermodynamical and other considerations that S-shaped curves are predicated. In the instance of elastic gels at low vapor pressure the adsorption of water by the colloid is of greater importance than the influence of ionic constituents present in the gel; with increasing vapor pressure the adsorptive effect of the colloid becomes relatively insignificant as compared with that due to the effect of the ionic constituents in lowering the vapor pressure. The change in a biocolloid is thus the sum of the water-binding capacity of the colloid itself and of the ions which are present in the mass. In consequence, S-shaped curves result and curves of this type have been reported in the literature for a large number of biocolloids.

Discussion

This study, conducted under carefully controlled conditions, shows that flour responds rapidly to changes in relative humidity and that the rate of loss in moisture greatly exceeds the rate of regain. Substantiation in this regard is afforded by similar observations of Fairbrother (1929), who suggested that even at normal temperatures the drying of flour apparently permanently reduces its hydration capacity. Not only does the apparent hygroscopicity decrease with increasing temperature at which the hydration capacity is determined but even at constant temperature the hygroscopicity of a particular flour at a particular relative humidity is not a definite quantity, but depends upon its past history. In view of our knowledge of the effect of age on the hydrophilic properties of biocolloids, it would furthermore be surprising if a given wheat flour stored at a definite moisture and temperature showed constant hygroscopic equilibria values over any extended period of time. The studies reported in this paper were conducted with only one flour and there is reason to believe that flours of varying chemical composition

under precisely similar conditions of storage would yield different hygroscopic values. The data of Coleman and Fellows (1925), Fairbrother (1929), and Pap (1931) reveal that different wheats show considerable variation in their hygroscopicity and on storage of different wheats of varying moisture content together, equality in moisture content is not attained. In view of these considerations and the differences in experi-

TABLE VII

HYGROSCOPICITY OF WHEAT FLOUR AS REPORTED BY DIFFERENT WORKERS

Relative humidity	Flour moisture content				
	Bailey (1920)	Fairbrother (1929)	Anderson (1937)	Anker, Geddes and Bailey	
				Highest values [1]	Lowest values [2]
%	%	%	%	%	%
30	5.2	9.4	—	9.7	8.5
40	6.5	9.7	—	11.1	9.9
50	8.0	10.7	12.0	12.3	11.1
60	9.8	12.8	13.7	13.2	12.3
70	12.1	14.0	15.4	14.5	13.7
80	15.0	15.7	—	16.3	15.8

[1] Tests at 25°C (77°F) with flour containing 12.2% moisture.
[2] Tests at 37°C (98.6°F) with flour containing 12.2% moisture.

mental technique, it is not surprising that wide discrepancies exist in the hygroscopicity of flour as reported by different workers. The available data known to the authors for selected humidities are summarized in Table VII. Approximate hygroscopic equilibria may also be computed from the storage tests; the best bases for this purpose would appear to be the mean values for the 5- and 10-pound paper sacks for those storage periods where the changes in weight are reasonably stable. These are compiled in Table VIII and compared with the equilibrium

TABLE VIII

COMPARISON OF HYGROSCOPIC EQUILIBRIA FROM STORAGE TESTS ON 5- AND 10-POUND PAPER AND COTTON SACKS AND LABORATORY SCALE STUDIES

Relative humidity	Approximate equilibrium moisture			Storage periods included in computation of second column
	Storage tests at 21.1–21.7°C	Laboratory tests at 25°C, flour at		
		12.2% moisture	14.7% moisture	
%	%	%	%	
36	9.8	10.5	9.7	5 to 10 weeks
45	11.3	11.7	10.9	7 to 9½ weeks
59	13.2	13.1	12.8	5 to 8 weeks
72	14.4	14.7	14.5	5 to 9½ weeks

values determined at 25°C (77°F) for the flours at 12.2% and 14.7% moisture as read from Figure 2. In view of the differences in temperature and original moisture content of the flour in the storage trials and laboratory tests, the agreement is perhaps all that could be expected, with the exception of the values for 36% relative humidity. It will be recalled that the flour used in the storage trials at this humidity level was first exposed to 30% and 65% relative humidity.

The observations made in these studies are of considerable practical significance to millers. Data collected from a large number of mills located in various sections of the United States concerning the moisture content of flour at the time of packing are now being studied in detail

TABLE IX

PERCENTAGE OVERPACKING REQUIRED TO INSURE 100% OF THE REQUIRED WEIGHT AT VARYING RELATIVE HUMIDITIES WITH FLOUR PACKED AT MOISTURE CONTENTS OF 13.0% TO 15.0%

Relative humidity	Flour moisture at equilibrium	Percentage overpacking required to provide full net weight for flours packed at following moisture contents:				
		13.0%	13.5%	14.0%	14.5%	15.0%
%	%	%	%	%	%	%
10	5.9	8.2	8.8	9.4	10.2	10.7
20	8.1	5.6	6.2	6.9	7.6	8.1
30	9.6	3.9	4.5	5.1	5.8	6.4
40	11.1	2.2	2.8	3.4	4.0	4.6
50	12.2	0.9	1.5	2.1	2.7	3.3
60	13.2	−0.2	0.3	0.9	1.5	2.1
70	14.5	−1.7	−1.2	−0.6	0.0	0.6
80	16.5	−4.0	−3.5	−2.9	−2.4	−1.8

to determine the regional and seasonal variations in the moisture content of different types of flour. A survey of the data shows that flours manufactured for the family trade range in moisture content, as milled, from approximately 13.0% to 14.0% moisture, the average being probably closer to the higher value. These flours are normally packed in small-sized paper and cotton sacks and hence are extremely susceptible to changes in weight due to atmospheric conditions. If we assume an average moisture content of 13.5% for the flour as packed, our hygroscopicity data indicate that the flour would have to be maintained at a relative humidity of at least 60% to prevent loss in weight. In many districts of the United States the humidity in stores and heated warehouses may fall much below this value for considerable periods, especially in the colder parts of the country during the winter months. The results of our studies and those of Fairbrother (1929) show that moisture will be lost rather rapidly but will be slowly and incompletely re-

gained upon re-exposure to higher humidities, thus likely resulting in a permanent weight loss.

According to the Federal Standards, flour may be packed at moistures up to 15.0% as determined by the vacuum or 130°C air-oven methods. It is therefore of interest to calculate the extent of overpacking flour which would be necessary to ensure 100% of the required weight at varying relative humidities for flours packed at moisture contents of 13.0% to 15.0%. For the purpose of these calculations, which are summarized in Table IX, the highest hygroscopic equilibria values obtained in our studies have been employed, namely those obtained at 25°C with flour at 12.2% initial moisture. These figures are only illustrative. Lower hygroscopicity values would result from exposure at higher temperatures and probably also with increasing age of the flour. In view of the slow rate of regain and the probable permanent reduction in hydration capacity it would be essential, in order to ensure full net weight, to overpack sufficiently to provide for the lowest relative humidity and the highest temperature at which the flour is ever likely to be exposed. This would imply that a large proportion of the flour packed for the family trade would reach the consumer overweight.

Summary

Studies have been conducted of the changes in net weight and moisture content of an 83% patent flour (commercially milled for the family trade from a blend of hard red spring and hard red winter wheats) and containing 13.3% to 13.4% moisture as packed in 5-, 10-, and 24½-pound paper and cotton bags and stored in air-conditioned cabinets maintained at 70°–71°F (21.1°–21.7°C) and relative humidities of 36, 45, 59, and 72% respectively with an accuracy of approximately ± 1°F and 2% to 3% relative humidity.

Rate of change in weight was relatively rapid during the first several days of storage, the actual rate for any one type of container depending upon the magnitude of the difference between the original moisture content of the flour and the moisture content which would finally be attained at the existing relative humidity.

Initial response in weight to any storage condition depended upon the size and kind of package, the rate of change decreasing with the package size and being greater for cotton than for paper sacks. As the storage period was prolonged the cumulative percentage changes in weight for the various containers tended to equalize and approach a common value. No definite equilibrium was obtained even after eight to ten weeks' storage.

Rate of moisture loss on exposure to low relative humidity is much more rapid than the rate of regain; the partial drying of flours even at atmospheric temperatures apparently permanently reduces their hydration capacity.

The approximate moisture equilibria obtained in the storage trials at 70°–71°F (21.1°–21.7°C) for 36, 45, 59, and 72% relative humidity were 9.8, 11.3, 13.2, and 14.4% moisture respectively as determined by the 130°C air-oven method.

Laboratory studies of the hygroscopic equilibria of the same flour brought to initial moistures of 6.5, 12.2, and 14.7% conducted at 25°C (77°F) over the range of 10% to 80% relative humidity revealed that the equilibrium is influenced either by the initial moisture content of the flour or by the environmental conditions to which the flours were exposed during conditioning. Flour at 6.5% moisture had the lowest hygroscopicity and at 12.2% moisture the highest, the differences at corresponding humidities being approximately 1.0%.

Hygroscopic equilibrium of flour at constant relative humidity varies with temperature. Comparative tests at 25°C (77°F) and 37°C (98.6°F) gave differences up to approximately 1.0% at certain humidities.

An S-shaped curve best expressed the relation between relative humidity and hygroscopicity, and is in accord with theoretical considerations of the water relationships of colloids.

A given flour does not possess a definite hygroscopicity, the hydrophilic properties being influenced by its past history.

The practical implications of these studies in relation to the changes in weight of packaged flour are discussed.

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THE EVALUATION OF FLUOROPHOTOMETERS TO BE USED IN THE THIOCHROME ASSAY FOR VITAMIN B_1 [1]

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The present investigation was undertaken for the purpose of establishing the reliability and precision that may be expected in the thiochrome assay of vitamin B_1 with available commercial fluorophotometers as far as instrument performance is concerned. Five fluorophotometers of varying design and construction were tested for: (1) time required to attain stable operating conditions, (2) linearity of response with quinine sulfate, thiochrome, and thiochrome in flour eluate, (3) reproducibility of readings, (4) sensitivity, (5) effect of line voltage fluctuations, (6) fatigue, and (7) rate of destruction of thiochrome.

Methods of Exciting and Measuring Fluorescence in Fluorophotometers

The general methods of investigating fluorescence in biochemical substances have been reviewed in detail by Dhéré (1937). In order to excite fluorescence, the substance, generally in solution, must be illuminated with light of appropriate wave length. For the fluorophotometric assay of thiochrome, the mercury arc lines in the ultraviolet at 365 mμ furnish satisfactory exciting radiation. High-pressure mercury arcs of the H-3 or H-4 type have strong lines in this region of the spectrum, and are customarily employed. It is not necessary that they be in quartz envelopes, as glass is sufficiently transparent at 365 mμ. Mercury arcs with quartz envelopes (*e.g.*, those of the "Uviarc" type) may, however, be used.

The radiation from all of these sources contains much energy in the visible part of the spectrum as well as in the ultraviolet, and it is necessary to eliminate the visible rays as efficiently as possible in order to prevent their effect from being added to that of the fluorescent visible light. For this purpose, a "primary filter," which transmits ultraviolet but absorbs visible radiation, is used. Several filters are available for this purpose. The four used in the instruments investigated are Corning Nos. 584, 586, 587, and 597, of which the transmission curves are shown in Figure 1. In general, those which transmit more of the ultraviolet also transmit more radiation at the

[1] Contribution No. 192 from the Department of Biology and Public Health, Massachusetts Institute of Technology.

blue end of the visible spectrum—hence increase in exciting energy is attained only with sacrifice of spectral purity.

The photocell for measuring the intensity of the fluorescent light is customarily placed at the side of the cuvette containing the fluorescing solution so as to minimize the amount of radiation falling on it from the primary ultraviolet beam. Nevertheless, the primary energy scattered into the photocell is of sufficient magnitude so that its effect must be reduced, as far as practicable, by "secondary filters" which transmit the fluorescent light freely but absorb the primary ultraviolet.

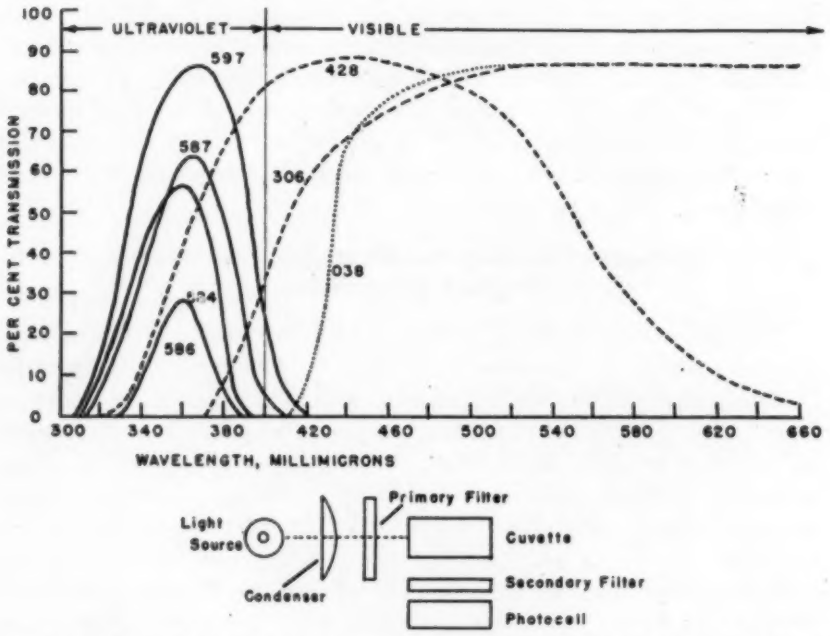

Fig. 1. Set-up for photoelectric fluorophotometry, and transmission curves of filters used in the thiochrome assay of vitamin B_1.

One of two alternative secondary filter systems is ordinarily employed. The first consists of a Corning Type 038 followed by a Type 428. The 038 absorbs efficiently all ultraviolet of wave length shorter than about 410 mμ, but the absorbed ultraviolet causes this filter to emit yellow fluorescent light. The Type 428 filter absorbs part of the fluorescent radiation from the 038 filter, but transmits the fluorescent radiation from thiochrome. The combination effectively eliminates most of the scattered ultraviolet from the primary beam. If, instead of this combination, a Type 306 or 038 Corning filter is used alone, somewhat greater transmission of the fluorescent light from the

solution is obtained, but this is diluted by the entire fluorescent light from the secondary filter. The absorption curves of the secondary filters customarily used are shown in Figure 1, and the primary and secondary filter combinations recommended by the manufacturers for the various instruments investigated are shown in Table I.

TABLE I

INSTRUMENT MANUFACTURERS' RECOMMENDED FILTER COMBINATIONS

Instrument	Light source	Primary filter	Secondary filters (in order from cuvette to photocell)
A	H3	Corning 584	Corning 038, 428
B	"Uviarc"	Corning 586	Corning 038, 428
C	H4	Corning 597	Corning 306
D	H4	Corning 587	Corning 038, 428
E	H4	Corning 587	Corning 038, 428

Photocells and Electrical Circuits Used in the Fluorophotometers Investigated

Two types of photocells are employed in existing fluorophotometers: barrier layer and photoemissive cells. The former have the advantages of being self generating (that is, of needing no external battery or source of emf) and of having high sensitivity. They are, however, subject to fatigue, especially if exposed to considerable infrared radiation, and they give linear response in relation to light intensity only when used with low resistance loads and at low light levels. Photoemissive cells have comparatively linear response with high resistance loads and are free from fatigue, but they must be used in connection with accessory electronic amplifiers. Cells of either type may be employed satisfactorily for fluorescence measurements. When barrier layer cells are used, the investigator should be aware of their liability to fatigue. Because of the low light levels characteristic of fluorescence, however, fatigue is only of consequence in fluorescence measurements when instruments are used continuously for extended periods of time.

In the simplest circuits, a barrier layer photocell is directly connected to a sensitive galvanometer, and the galvanometer deflection is calibrated in terms of solution concentration. A variable shunt is provided for adjusting the sensitivity of the galvanometer, and a battery and potentiometer may be employed for surpressing the zero, so that the readings of the blank are zero. In order to obtain greatest linearity of response, the galvanometer should have a resistance of less than 1,000 ohms (Lange, 1938). The galvanometer sensitivity required can be calculated from our measurements, which indicate

that with thiochrome equivalent to a total of 0.1 μg of thiamin in the cuvettes of the various fluorophotometers investigated, the light intensity on the photocells is of the order of 2×10^{-5} lumens per square centimeter of photocell surface. The barrier layer cells usually employed have sensitivities of the order of 500 microamperes per lumen and surface areas of about 10 square centimeters. For readings of 20-scale divisions for 0.1 μg of thiamin it is therefore necessary to use galvanometers having sensitivities of the order of $2 \times 10^{-5} \times 10 \times 500/20 = 5 \times 10^{-3}$ microamperes per scale division.

TABLE II

PHOTOCELLS AND CIRCUITS EMPLOYED WITH FLUOROPHOTOMETERS TESTED

Instrument	Photocells	Type of circuit
A	1 barrier layer	Cell directly connected to sensitive, low-resistance galvanometer.
B	3 barrier layer (2 for fluorescent pickup, 1 for balancing)	Current balancing null circuit (Brice).
C	2 barrier layer	Potentiometric null circuit.
D	1 barrier layer	Potentiometric null circuit using battery for potentiometric voltage.
E	1 photoemissive (RCA 929)	Single stage electronic amplifier and output meter.

Barrier layer photocells may also be used in circuits in which the current or potential developed by the photocell is balanced against an opposing current or potential from a battery or from a comparison photocell placed in a part of the light beam. Various circuits have been devised for this purpose, some of which are especially designed to cause the photocells to operate into low resistances at balance, so that their output will be approximately a linear function of light intensity. Such circuits have been reviewed by Müller (1939). One advantage of using two photocells in a null circuit is that variations in readings due to fluctuations in line voltage (which are, of course, reflected as fluctuations in intensity of the light source, and therefore as changes in fluorescent light) may be eliminated almost completely in this way.

When photoemissive cells are used, the current from them may be amplified by a single-stage electronic amplifier and measured in terms of amplifier plate-circuit current.

It is not the purpose of the present paper to give details of circuit design. The photocells and general types of circuits used in the instruments investigated are summarized in Table II.

Time Required to Attain Stability of Operation

The mercury-vapor lamps used as ultraviolet sources in fluorophotometers require considerable time to attain their full operating temperature. During this preliminary period, the intensity of their emitted radiation constantly increases. It is important, therefore, to permit full operating intensity to be attained before readings are taken. The following experiments were conducted to determine how long an initial heating period is required before the maximum intensity level is reached.

Using two instruments (A and E) which did not employ balancing photocells, a solution of quinine sulfate solution (concentration 0.27 μg per ml) was added to the cuvettes,[2] and the instruments were adjusted to give full-scale deflection after a stabilizing period of at least half an hour. The lamps were turned off and allowed to cool to room temperature. They were then turned on again and readings were taken at intervals of one minute from the moment of lighting until stability had been attained.

The results for instrument A, incorporating an H-3 type mercury arc, and for instrument E, employing an H-4 type mercury arc, are shown in Figure 2. It is evident that in both instances full operating intensity was reached before ten minutes had elapsed. In the case of the H-4 lamp, the intensity rose somewhat more quickly, reaching its maximum in six minutes. These curves should not be taken as quantitative comparisons of the characteristics of H-3 and H-4 lamps, however, as the rate of temperature rise in each instance depends somewhat upon the type of lamp housing and ventilation employed. In the cases illustrated, the H-4 lamp was cooled by a fan, whereas the H-3 was not.

In order to see whether the rise of intensity would be more rapid if the lamps were not cooled completely to room temperature before relighting, the H-4 current supply was turned off at ten minutes and immediately turned on again. It is a characteristic of lamps of this type that they do not relight immediately when hot. It was found that the H-4 lamp would relight after a cooling period of about two

[2] The greater photochemical stability of quinine sulfate solutions than of thiochrome solutions makes it advantageous to use the former as the fluorescing material in instrument tests. It seemed desirable, however, to use quinine sulfate at concentrations easily translatable into thiamin equivalents. The quinine sulfate secondary standards recommended by the various instrument manufacturers ranged in concentration from 0.20 to 0.27 μg per ml. It was found in our tests that the higher limits of these concentrations gave a fluorescent response with the particular instruments tested approximately equivalent to that of 1 μg of thiamin oxidized and taken up as thiochrome in 10 ml of butanol, that is, of 0.1 μg of thiamin per ml of final thiochrome solution. It should be noted that, provided a minimum volume of thiochrome solution is used sufficient to fill the cuvettes to the proper height, the response of fluorophotometers is a function of the final thiochrome concentration only, and 1 μg of thiamin oxidized and taken up in 10 ml of butanol gives the same response as 1.5 μg oxidized and taken up in 15 ml. Since we found that a 0.27 μg quinine sulfate solution in 0.1*N* H_2SO_4 gave a fluorescent response equivalent to that of a thiochrome solution corresponding to 0.1 μg of thiamin per ml of final thiochrome solution, this concentration of quinine sulfate was adopted as the secondary standard for use in our tests. The 0.27 μg per ml quinine sulfate solution was made up by 40-to-1 dilution of a stock solution of 10.8 μg per ml.

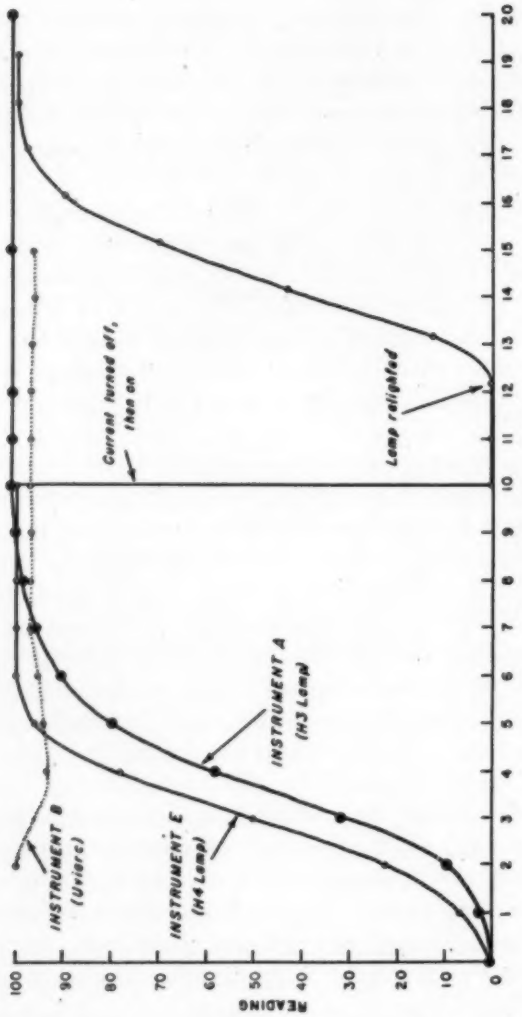

Fig. 2. Heating-time tests using quinine sulfate at 0.27 μg per ml in 0.1*N* H_2SO_4.

minutes in the particular lamp housing used. As shown by the figure, however, the rate of increase in intensity after relighting was almost identical with that found for the same lamp started at room temperature.

In the case of fluorophotometers in which line voltage fluctuations are balanced by a second photocell illuminated by a part of the radiation from the lamp, stability of operation might be expected as soon as the lamp had attained sufficient intensity for satisfactory null readings to be obtained with the potentiometer. Tests of the time required to attain stability for a fluorophotometer of this type (instrument B) are shown in Figure 2. It was not possible to balance the fluorophotometer until two minutes after the lamp had been turned on. The null reading then obtained was at 100 on the potentiometer scale, the value to which the instrument had been adjusted when the lamp was fully heated. During the next two minutes, there was a slight decrease in readings, and subsequently a slow rise until after seven minutes the readings remained constant. While the initial heating period makes much less difference in the readings of instruments of this type, it is evident that it is better, even with balanced circuits, to allow time for stable operating conditions to be attained.

It appears from these results that the initial "warming-up" periods of 30 minutes to one hour used by many workers is unnecessarily long. A heating period of 10 to 15 minutes seems entirely adequate for mercury arcs of the types usually employed in fluorophotometric investigations.

Linearity of Response, General Considerations

It is desirable that the readings obtained be directly proportional to the concentration of the fluorescing material. If a linear relationship obtains between readings and concentration, the calibration of the instrument is considerably simplified and the accuracy with which interpolation can be made between calibrated points is increased. If readings are a strictly linear function of concentration, one has but to obtain two values, r_1 and r_2, for two known concentrations, c_1 and c_2, following which the concentration X of any unknown solution may be determined from the corresponding net reading (minus the blank) R, by simple proportion.

$$X = R\frac{c_1 - c_2}{r_1 - r_2}.$$

Linearity of response may be achieved if (1) the intensity of fluorescent light is directly proportional to the concentration of fluorescing material, and (2) the response of the instrument is a linear function

of the light incident on its photocells. In order for the first condition to be met, it is necessary that the concentration of the solution be sufficiently low so that it absorbs inappreciably either exciting radiation or fluorescent light. For the second condition to be met, it is necessary that the electrical design of the instrument be such that the fluorescent light is converted into an electrical current or voltage directly proportional to it. This may be achieved in the case of cells of the photoemissive type by the use of carefully designed amplifier circuits, and in the case of those of the barrier layer type by careful choice of cells and by using them only with low resistance loads and operating them at low light-intensity levels.

The limiting concentration above which there is appreciable departure from linearity of fluorescent light intensity as a function of concentration is also related to the shape and size of the cuvette. The longer the path of incident and fluorescent light through the cuvette, the greater the absorption of light will be. Doubling the path length d is equivalent to doubling the concentration c insofar as influence on absorption is concerned, as is evident from the following expression (the Lambert-Beers absorption law) for the absorption of light of a given wave length:

$$I_d = I_0 e^{-kcd},$$

where I_0 and I_d are respectively the light intensity before and after traversing a path length d through the solution, e is the base of Napierian logarithms, c is the concentration, and k is the absorption coefficient of the solution at the particular wave length in question, in units corresponding to those employed for c and d. It would appear, therefore, that to increase the range of linearity, the smallest possible cuvettes should be used. On the other hand, reduction in cuvette size, with the concentration held constant, results in decrease in the light available for operating the photocells. There are, therefore, practical limits beyond which this method of remedying the situation cannot be carried.

Linearity of Response with Quinine Sulfate

The linearity of response was tested with two series of dilutions of quinine sulfate in 0.1N H_2SO_4, one ranging in concentration from 0.027 to 0.27 μg per milliliter (corresponding to thiamin hydrochloride equivalent to 0.01 to 0.1 μg per ml of thiochrome solution) and the other ranging from 1.08 to 10.8 μg per ml (corresponding to thiamin hydrochloride equivalent to 0.4 to 4.0 μg per ml of thiochrome solution). In both instances, the sensitivities of the instruments were adjusted, when possible, to give approximately full-scale reading for

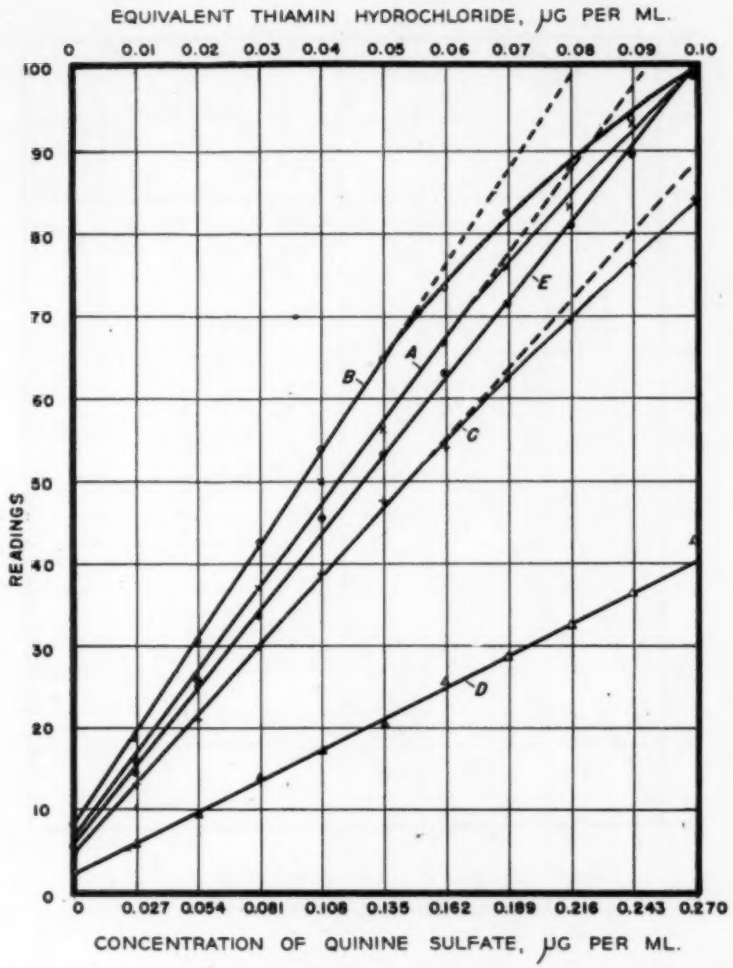

Fig. 3. Tests of linearity with quinine sulfate, 0 to 0.27 µg per milliliter.

the maximum concentration employed. The results for the lower concentration range are shown in Figure 3 and those for the higher range in Figure 4. All readings are given on the basis of 100 for full scale. All of the instruments tested gave approximately linear response up to a level of 0.162 µg of quinine sulfate per ml (corresponding to 0.06 µg of thiamin hydrochloride per ml of thiochrome solution) and two of them (D and E) gave linear response to considerably higher levels. In the higher concentration range, the departure from linearity was considerably more marked (Fig. 4), but since these levels corresponded to greater concentrations of thiochrome than one would

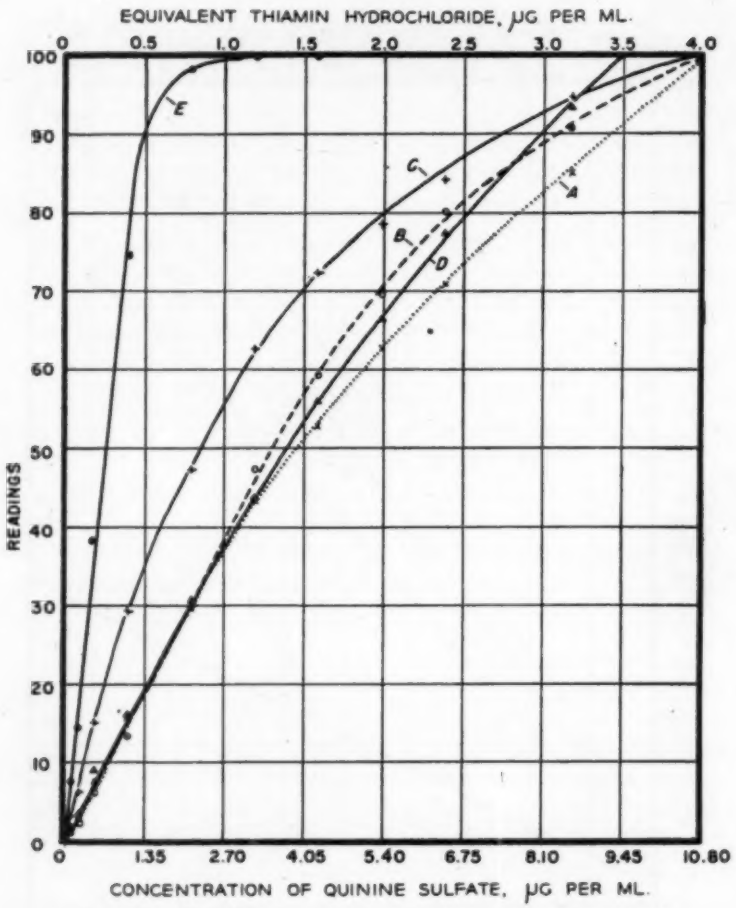

Fig. 4. Tests of linearity with quinine sulfate, 0 to 10.8 µg per milliliter.

usually wish to test, the lack of linearity under these conditions is not particularly important.

With one instrument (C), which employs a quinine sulfate comparison standard to excite the balancing photocell, it was found necessary to remove this standard between readings in order to avoid errors due to photochemical deterioration of the standard.

Linearity of Response to Graded Amounts of Thiochrome

To test the linearity of response with thiochrome, a thiochrome solution was prepared as follows:

One milliliter of a stock solution of thiamin prepared in accordance with USP XI procedure (2nd supplement, pp 131–2) and containing 500 µg per ml was diluted to 100 ml with 0.1*N* sulfuric acid. Four ml

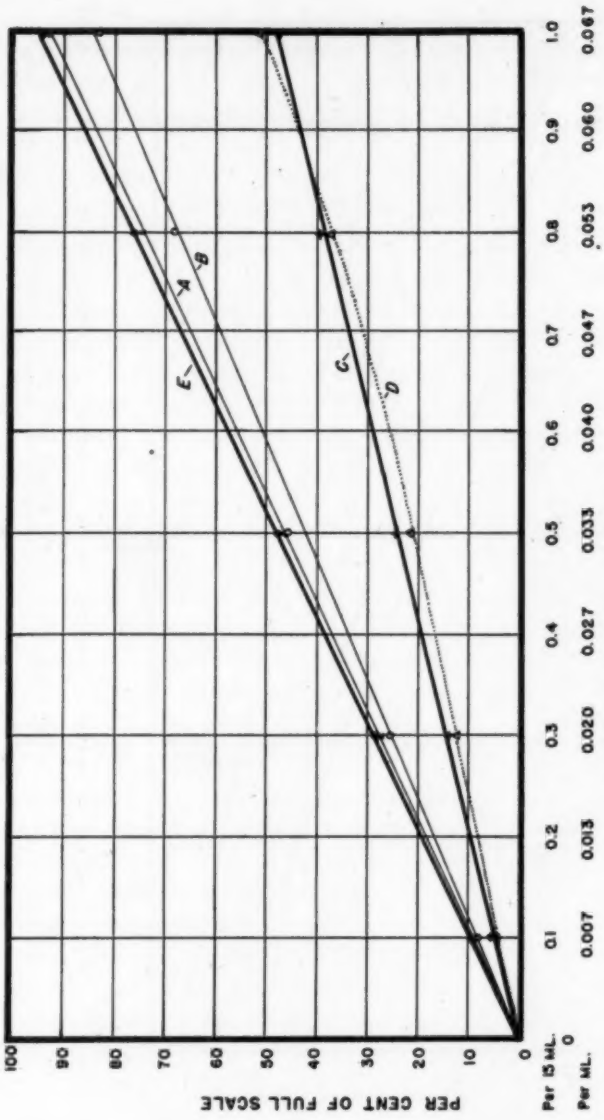

Fig. 5. Tests of linearity with thiochrome in butanol.

of this dilution was diluted to 100 ml with 25% potassium chloride solution in 0.1*N* hydrochloric acid. 5.0-ml aliquots from this KCl dilution (each aliquot containing a total of 1.0 μg of thiamin) were put into two separatory centrifuge tubes. To the first, 3 ml of a 15% sodium hydroxide solution containing 0.03% potassium ferricyanide were added and quickly mixed. To the second, 3 ml of 15% sodium hydroxide were added in the same way. Fifteen ml of distilled iso-

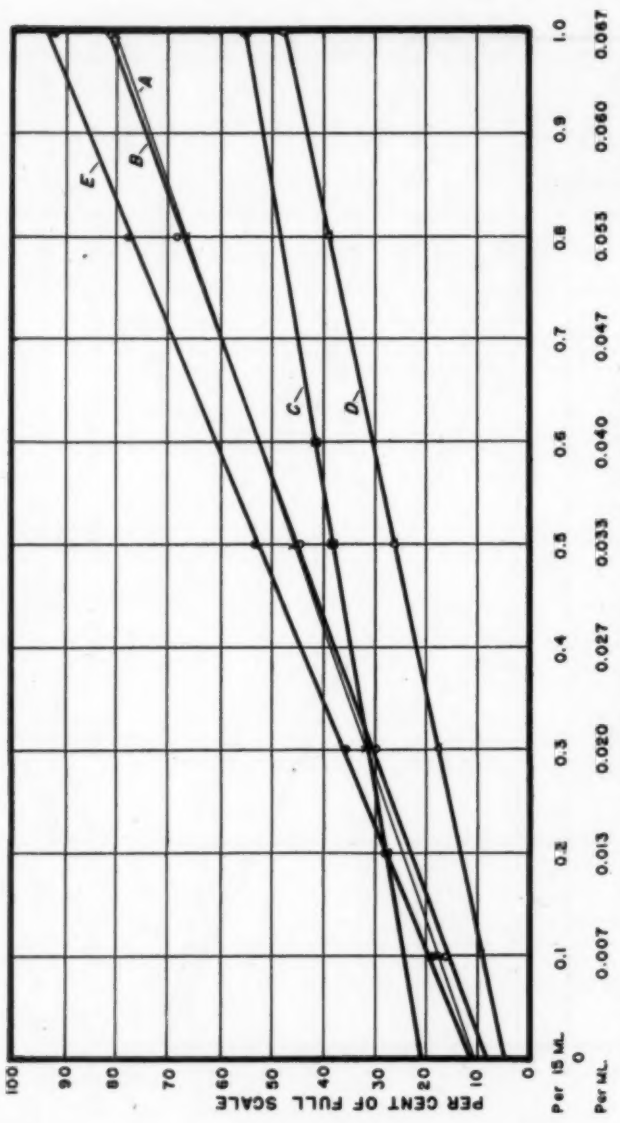

Fig. 6. Tests of linearity with thiochrome in flour eluate.

butanol were added to each, the tubes stoppered, shaken vigorously for 1½ minutes, then centrifuged at 500–600 rpm for ¾ minute in upright position. The stoppers were removed and the lower aqueous layer discarded. Two grams of anhydrous sodium sulfate was then added to each tube, the tubes shaken vigorously for a moment, and the contents poured into containers appropriate for reading of the instruments.

A series of solutions prepared from this dilution of the stock thiamin solution and containing graded levels of thiamin was oxidized, then tested on each instrument (Fig. 5). The response was approximately a linear function of concentration in each case.

Linearity of Response to Graded Amounts of Oxidized Thiamin Dissolved in Flour Eluate

The experiments with thiochrome were repeated using a composite extract of flour eluted from exchange zeolite with 25% potassium chloride. These eluates were prepared from four samples of flour in accordance with the procedure outlined by Hennessy (1941) with the exception that the flour solution contained 1.7% rather than 1.22% of sodium acetate.

The readings obtained with graded amounts of thiochrome in flour eluate are shown in Figure 6. All instruments responded in direct proportion to concentration.

The data of Figures 4, 5, and 6 indicate that all five instruments can reliably measure thiochrome in a cuvette or tube in concentrations corresponding to between 0.1 and 1.0 μg of thiamin hydrochloride per 15 ml of thiochrome solution. They indicate, further, that all instruments can rather accurately measure as little as 0.1 μg of thiamin per 15 ml of thiochrome solution in pure solution or in the presence of flour eluate.

Reproducibility of Readings

The reproducibility of readings was tested by making repeated determinations on a series of dilutions of quinine sulfate in 0.1N sulfuric acid ranging in concentration from 0.027 to 0.27 μg per milliliter. The instruments were adjusted, so far as possible, to give approximately full-scale reading with the dilution of maximum concentration. The line voltage was kept constant at 110 $\pm$ 0.25 volts.

The purpose of these experiments was to determine the magnitude of deviations (such as those due to zero drift of meters, variations in the setting of potentiometers at the null point, errors in estimating fractions of a scale division, etc.) which have a magnitude independent of the intensity being read. These may be expressed as percentages of full-scale deflection, or as fractions of a scale division. For the data shown in Table III, the deviations from the means were calculated for 20 readings. The table shows for each instrument the maximum average, and root mean square (standard) deviations. The root mean square deviation, σ, where

$$\sigma = \sqrt{\frac{\Sigma(M - x)^2}{n}}$$

M is the mean of readings, x is the value of each individual reading, and n is the total number of readings (in this case 20), is useful because, if we assume that the frequency of errors of various magnitudes follows the "normal curve of error," one can calculate from the standard deviation, σ, the probability of any reading deviating by more than a particular amount from the mean due to inherent errors of the instrument. Thus there is a 50% chance of readings deviating more than 0.675σ from the mean. This value is known as the probable error, P, and is shown in the last column of Table III.

TABLE III

PROBABLE ERRORS OF READINGS IN FRACTIONS OF A SCALE DIVISION
(On basis of full scale = 100 divisions)

Instrument	Deviation from mean (20 values)			Probable error of readings, P [1]
	Maximum	Average	Standard	
A	±1.0	±0.63	±0.68	±0.46
B	1.9	1.06	1.17	0.79
C	1.7	1.00	1.04	0.70
D	2.3	0.95	1.09	0.74
E	1.0	0.44	0.51	0.34

[1] The probable error is 0.675 times the standard deviation, and is the limit of deviation within which 50% of random readings may be expected to lie.

Sensitivity

All instruments were adjusted to give as large a reading as possible with quinine sulfate at 0.027 μg per ml (equivalent to 0.01 μg thiamin hydrochloride per ml of thiochrome solution). From the readings R so obtained, the readings of the corresponding blanks B (cuvette filled with $0.1N$ H_2SO_4) were subtracted. These results were used to calculate the sensitivity S in μg per ml per scale division (Table IV).

TABLE IV

TESTS OF SENSITIVITY WITH QUININE SULFATE

Instrument	Micrograms per milliliter per scale division at maximum sensitivity [1]	
	Quinine	Thiamin equivalent [2]
A	0.0014	0.00051
B	0.0013	0.00047
C	0.00092	0.00034
D	0.0078	0.0029
E	0.00078	0.00029

[1] Using the filters recommended by the manufacturers for thiochrome determinations (see Table II).
[2] Equivalent thiamin hydrochloride per ml of thiochrome solution.

$$S = \frac{0.027}{R - B}.$$

By multiplying the probable error in scale divisions by the sensitivity per scale division one obtains the probable error in reading the material in question at maximum instrument sensitivity. Such values for deviations of one to four times the probable error (corresponding to chances of 50% to 0.7%) are shown in Table V.

TABLE V

PROBABLE INSTRUMENT ERRORS IN TERMS OF THIAMIN

Instrument	Error in μg of thiamin per ml of thiochrome solution[1] for various multiples of probable error *P*			
	P	2*P*	3*P*	4*P*
A	±0.00023	±0.00046	±0.00069	±0.00092
B	0.00037	0.00074	0.00111	0.00148
C	0.00024	0.00048	0.00072	0.00096
D	0.0021	0.0042	0.0063	0.0084
E	0.00010	0.00020	0.00030	0.00040
	Probable chance of occurrence of error of magnitude shown			
	50%	17.7%	4.3%	0.7%

[1] Equivalents of the quinine sulfate used in the determinations.

Effect of Line Voltage Fluctuations

In order to investigate the influence of line voltage fluctuations on the readings obtained with the various instruments, all instruments were adjusted to full scale deflection with the quinine sulfate at a concentration of 0.27 μg per ml in their cuvettes. Solutions of half this concentration were then substituted, and readings were taken at line voltages from 100 to 120. The voltage was regulated by means of an autotransformer (General Radio "Variac").

In all instances, the change in reading obtained was approximately a linear function of the change in line voltage (Fig. 7). In the case of instruments not employing balancing photocells to compensate for line voltage fluctuations, voltage changes caused variation in readings of the order of 2% to 4% per volt. In the case of instruments having balancing photocells, variations of the order of 0.1% to 0.15% per volt were obtained.

Values were also obtained after compensating line voltage fluctuation in the unbalanced instruments with a line voltage regulating transformer (Raytheon). Under these conditions fluctuations were reduced to about the same order as those experienced with balanced-circuit instruments. It appears that this method of line voltage control is satisfactory, if the frequency regulation of the supply line

is good. It is well known that changes in the frequency of the supply current lead to changes in the output of voltage regulating transformers. Consequently, the transformer compensation method should be used only in instances in which the line frequency regulation is adequate.

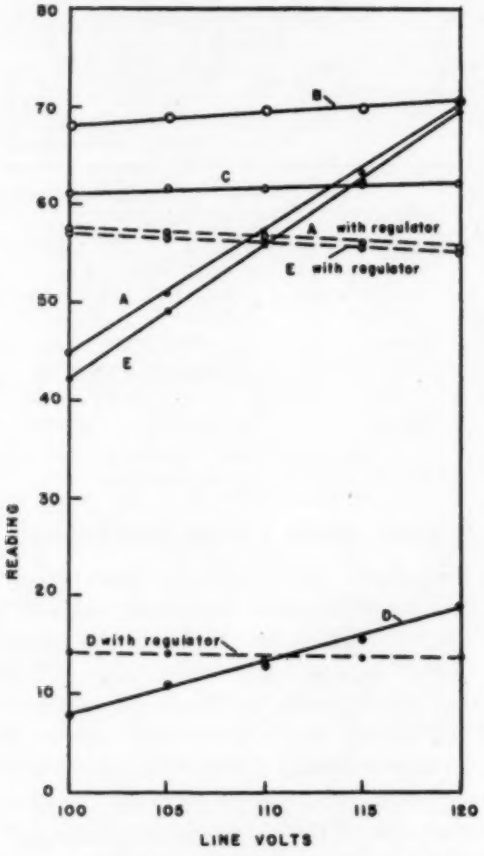

Fig. 7. Effect of line voltage variation on readings.

Relative Destruction of Thiochrome

The instruments were adjusted so far as possible to give full-scale readings with thiochrome in a concentration equivalent to 1.0 μg of thiamin hydrochloride per 15 ml of thiochrome solution in butanol. A fresh solution was then placed in each instrument, a reading was made as soon as possible, and subsequently $\frac{1}{2}$, 1, 2, 3, 5, and 8 minutes after insertion (Fig. 8).

In this experiment, instrument E was used as recommended; the button controlling the shutter was pushed down and the solution

exposed to radiation only when a reading was taken. The advantage of this procedure is shown by the results of this experiment. Similarly, the shutter in instrument A was opened only to take readings. The effect of leaving this shutter open continuously is shown by the lower curve for A, which indicates considerable thiochrome destruction. The small amount of destruction in instrument B is evidently the result of its use of a 586 type primary filter in combination with two photocells, which permits sufficient sensitivity to be obtained at low levels of illumination of the fluorescing solution.

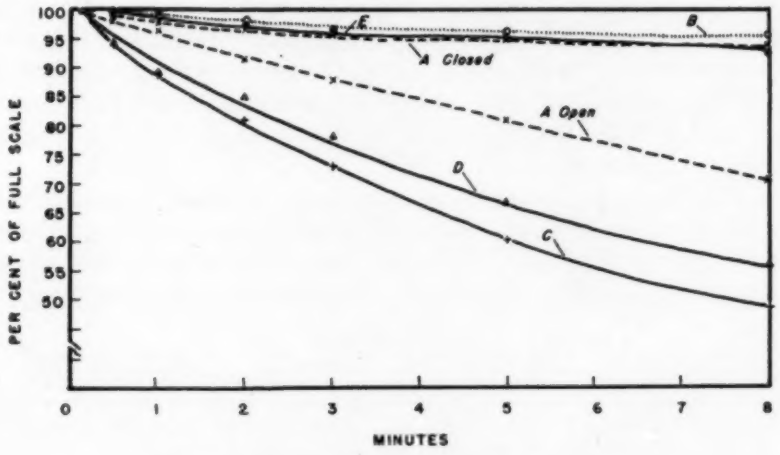

Fig. 8. Comparative rates of destruction of thiochrome.

In both instruments D and C the thiochrome is exposed to considerable radiant energy. As there is no means of protecting the test solutions while these instruments are in use, considerable thiochrome destruction normally occurs. While it is a good routine practice to standardize the procedure and make readings at a definite interval after placing the solution in an instrument in any case, this precaution seems imperative when using instruments C and D.

It was not possible to make a true zero reading since about 5 seconds (E), 10 seconds (A, B, D), and 15 to 20 seconds (C) elapsed with the various instruments before a proper reading could be made. However, if one is allowed to project the curves in Figure 8 back to a zero observation, it appears that perhaps 5% of the thiochrome is destroyed in instruments C and D before a reading can be made. This is not serious if an identical procedure is used in standardizing the instruments with pure thiamin. A very serious error can result if timing is not strictly observed.

Conclusions

The results of these investigations indicate that:

1. A heating period of 10 to 15 minutes is sufficient to attain stable operating conditions in the fluorophotometers tested.

2. All instruments tested have sufficient sensitivity, linearity, and reproducibility of readings to permit the evaluation of thiamin at levels of 0.1 to 1.5 μg per 15 ml of thiochrome solution with probable errors of the order of 0.0001 to 0.0021 μg of thiamin per ml of thiochrome solution.

3. The variation in readings due to line voltage fluctuations is of the order of 2% per volt at 110 volts in instruments without balanced photocell circuits. This is reduced to the order of 0.1% per volt in instruments employing balanced circuits, or in unbalanced instruments when used with line voltage regulating transformers on lines with good frequency regulation.

4. The rate of destruction of thiochrome in these instruments is insufficient to cause serious error in readings but in two instruments (C and D) it is high enough to make advisable the taking of readings at a constant time after introducing the sample into the instrument.

Acknowledgment

The assistance of the Research Corporation in supporting these investigations and the cooperation of the various equipment manufacturers in supplying instruments and accessory apparatus are gratefully acknowledged. Our thanks are due Mr. E. J. Josephson, Miss Lindy E. Marsh, Mr. Lloyd M. Mosher, Mr. C. V. Nelson, Mr. R. L. Sinsheimer, Mr. W. R. Slaunwhite, Jr., Mr. A. M. Webb, and Miss Elizabeth Weeks for their assistance during the course of the investigations.

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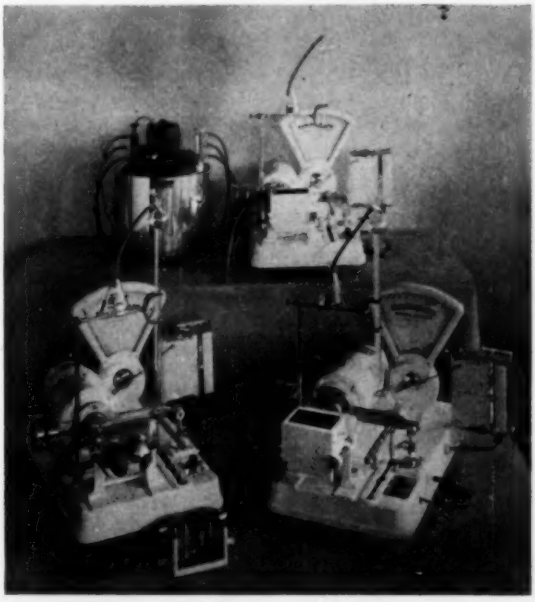

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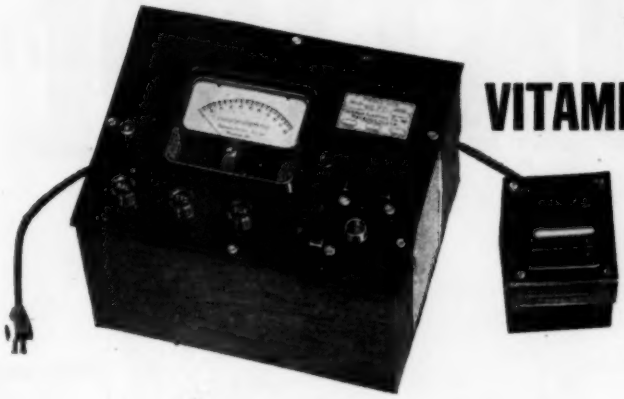

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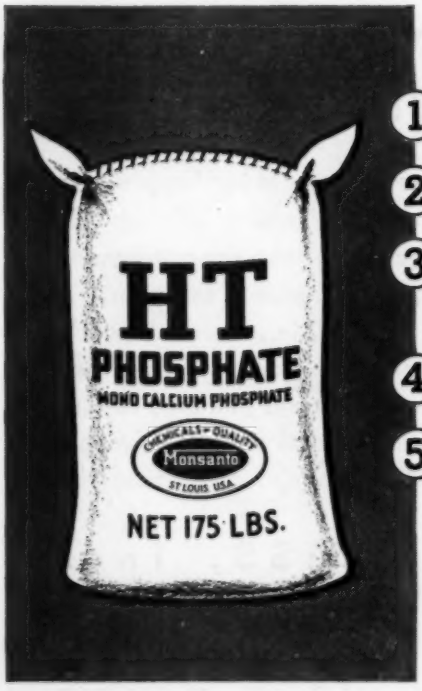

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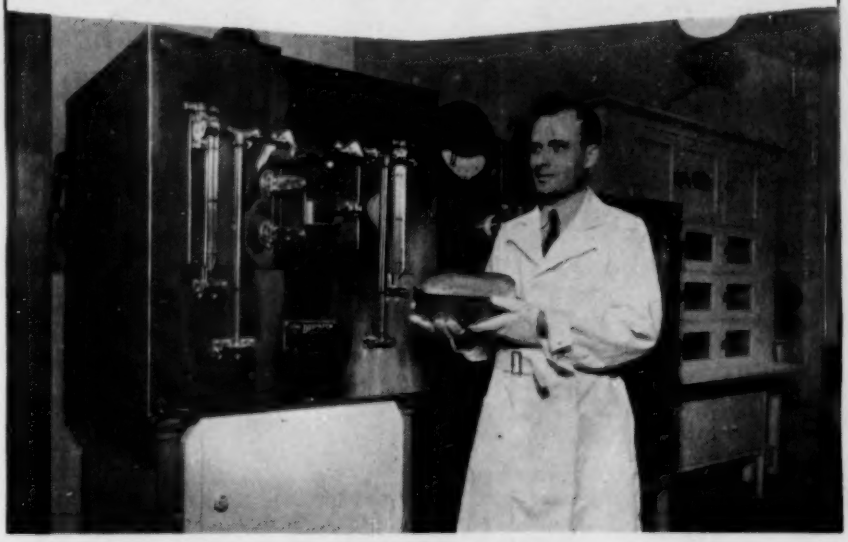

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